CONTROLS OF DEVELOPMENT IN COCKROACHES

by

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I. INTRODUCTION

All organisms have similar inherent goals--growth and propagation of the species. This genetically inherited goal-seeking behavior of organisms becomes increasingly complex among higher organisms. In the euploid protozoans each unicellular organism is its own germ line. However in the metazoa, the germ line is only one cell type of many. Each of these cell types has its own goal-seeking genome. The problem, then, which developmental biologists address themselves to is how these separate goal-seeking entities arise and how they are controlled in a way to achieve tissue goals, organ goals, and the organismic and social goals of growth and reproduction.

The complexity of the problem may be appreciated by counting the number of interactions at the organ level between components of a hypothetical organism. If we allow this hypothetical organism to have some essential organs such as an integument, muscular system, gastro-intestinal tract, kidney, liver, gonads, nervous system and endocrine system, this rather simple organism will have only 7 components. Between these 7 components there are 7!/(1! 1! 5!) = 42 interactions in which one component can have an effect upon another component.
Moreover, there are 7!/(2! 1! 4!) = 105 interactions in which 2 components can act jointly on a third component, and there are 7!/(3! 1! 3!) = 280 interactions in which 3 components can act jointly on a 4th component. All these interactions could take place in our hypothetical organism in a steady state equilibrium. Imposing development on this simple system adds the complication that each of these goal-seeking components may be changing, i.e. developing, in time and the interactions between these changing components may also be changing. It is the developmental biologist's task to chart these changes and interactions by careful observation and pointed experiments.

As emphasized above, the very question of development imposes complications on the system being studied. The problem of a constantly changing organism also creates a practical problem which students of relatively steady state complex systems do not encounter. This problem is the uncertainty of the actual physiological age of the animal.

Thus in working with metazoan development the biologist has to deal with these basic problems:

1. The large number of separate goal-seeking units (cells) and levels (tissue, organ, system, organism, society).

2. The large numbers of interactions between these units and levels.

3. The change in tim e of the goal-seeking units and levels which is inherent in development.

4. The uncertainty of the actual developmental stage of experimental material.

Any comprehensive model of development .of an organism m ust deal with the first three of the above problems. (c. f. Waddington, 1966). Any realistic model of development, to be used to simulate observable phenomena to aid in designing experiments on groups of animals, must also deal with the fourth problem, uncertainty of physiological age.

My thesis will involve the development of a realistic model of the control of development in cockroaches.

The cockroach was chosen for this study for a number of practical and theoretical reasons. The primary practical consideration was that cockroaches are extremely easy to culture. They are omnivorous and will thrive on a number of commercial laboratory chows. Their ease of culture is one of the contributing reasons why the two cosmopolitan roaches I use are household pests in almost every part of the world. The cockroach, because of its ubiquitous nature, has been the subject of a great deal of research in the past. Although most of this research on cockroaches has been concerned with physiology of the adult, a number of studies have dealt with problems of development. The model of hormonal control of insect molting proposed by Williams (1947) for the Saturniid silkmoth was confirmed in the American cockroach *Periplaneta americana* by Bodenstein (1953) who demonstrated that a substance from the brain of the cockroach is necessary to stimulate the prothoracic gland to produce the molting hormone. Thus the basic molting control axis in the cockroach has been established. Extrinsic controls of development have been useful in the past to enable researchers to manipulate their animals and also as a starting point to study developmental controls in general. A prominent example of this use of an extrinsic control in the field of physiological genetics is the study of enzyme induction and repression in bacteria by Jacob and Monod (1961). Pursuing the mechanism by which a substrate controls enzyme production they developed the operator model of control of protein synthesis. In an example concerning insect development, Wigglesworth (1934) used the extrinsic factor of feeding (Buxton 1930) to provide synchronous cultures of the blood-sucking bug *Rhodnius*. He investigated the mechanism of this extrinsic control of molting to give insect physiologists the first model of hormonal control of molting. This model did not recognize the prothoracic gland as the source of the hormone but rather cited the brainas its origin. This was later corrected by William's experiments on Saturniid silk moths and confirmed by Wigglesworth (1952) on *Rhodnius*. Williams himself was using Saturniid silkmoths because they underwent a resting stage, diapause, in the pupa. Diapause can be broken by environmental stimuli after which develop ment of the pupa continues toward the adult stage. If large numbers of pupae are released from diapause at the same time, a synchronously developing culture of animals results. Investigating this release from diapause led to William's model of hormonal control of molting. As shown by these examples, an extrinsic control of development gives an experimenter a practical as well as theoretical handle for studying controls of development in an animal.

Regeneration is such a handle for studying development in the cockroach. O'Farrell and Stock (1954) were the first to observe the effect of regeneration on the molting cycle of the German cockroach, *Blattella germanica*. They observed an all-or-none type of regeneration. If a leg was autotomized, i.e., reflexly lost at an autotomy point, prior to a certain critical period, the leg would be completely regenerated at the next molt and this regeneration would delay the molting cycle. If a leg was autotomized after the critical period, the molting cycle was not delayed and no regeneration would occur during this stadium. Wigglesworth (1964) in a subsequent review of progress in the study of insect development emphasized the possible importance the elucidation of this feedback mechanism could have to control theory.

The literature on regeneration in cockroaches extends back to debates in London Royal Society meetings in the 1840's on the possibility of its occurrence. Perhaps the first useful research in cockroach regeneration was done by Brindley (1877, 1898). Earlier workers had observed 4-segmented tarsi (rather than the usual 5 segments) among cockroaches collected from the wild. Since these legs were sometimes smaller than a normal leg, heated debate extended for half a century over whether they were regenerated legs or just developmental anomalies. Brindley (1897) compiled a vast amount of circumstantial evidence favoring the regeneration hypothesis, such as that newly-hatched

nymphs never have 4 segmental tarsi. Besides the circumstantial evidence, he observed the regeneration of 4-segmented tarsi in all instances where he removed 5-segmented tarsi in the first actual experiments of this kind to be performed. Brindley (1898) extended his studies of regeneration to observations on the effect of regeneration on molting cycle length. In a non-rigorous fashion, typical of the time, he thought he had indicated that:

1. "Reproduction" of a limb may take place in a shorter time in early stadia than in later stadia.
2. In the later stadia, "reproduction" of a limb may take place more rapidly in males than in females.
3. "Reproduction" of a tarsus occurs in a shorter time than more proximal reproduction.

Although point 2 has never been confirmed, points 1 and 3 have been established by a number of authors (O'Farrell and Stock 1954; Penzlin 1963).

More recently, O'Farrell and Stock have demonstrated that the critical period for regeneration is the same for all legs within a given animal. This was shown by taking off 2 legs. The four possible results would be:

Alternative	Leg 1	Leg 2
1	regeneration	regeneration
2	regeneration	non-regeneration
3	non-regeneration	regeneration
4	non-regeneration	non-regeneration

However only the first and last results were observed, which demonstrated that the critical period for regeneration was simultaneous for all limbs within an animal. The same conclusion was reached from similar evidence in the all-or-none regeneration of wing disc in the meal moth *Ephestia* (Pohley 1961). Double-limb regeneration delays molting a short time longer than single regeneration in both *Ephestia* and *Blattella*. O'Farrell and Stock (1953) also demonstrated that the all-or-none phenomenon in *Blattella* was associated with leg regeneration and not regeneration of other structures such as cerci or antennae.

All this research has led to speculation on the mode of feedback by which leg regeneration in the cockroach and wing disc regeneration in the meal moth delay molting.

The feedback of regeneration on the control of molting, resulting in an all-or-none regeneration of a limb, is not seen in all groups of insects. For instance the true bug *Rhodnius* (Luscher 1948) regenerates amputated legs gradually over a number of molting cycles; regeneration does not seem to affect the molting cycle. The more interesting cases, as far as control theory is concerned, are those such as leg regeneration in cockroaches and wing disc regeneration in the meal moth, in which regeneration does affect the control of molting. I therefore set out to study the mode of feedback by which leg regeneration in cockroaches alters the molting cycle.

The main drawback to using larval stages of cockroaches was discussed by Dennell (1956) and O'Farrell (1960). This lay in the fact that it was difficult to obtain large numbers of animals of a given

age. Dennell found it impossible to study the biochemical events leading up to ecdysis because he could not predict with sufficient accuracy when a group of cockroaches would undergo ecdysis. O'Farrell and Stock for the most part had to use first instar nymphs in their studies of regeneration which excused them from the impossible experiments of nerve section and ligation in those small animals. Although workable numbers of first instar animals of approximately the same age could be obtained by using animals which hatched on the same day, such synchronously started cultures quickly became progressively more asynchronous from instar to instar. Bodenstein (personal communication) also stated that American cockroach nymphs, started out synchronously as hatch]ings, were synchronous enough for his purposes for the first four instars, but subsequent instars were too asynchronous in their development to be useful as groups. In order to obtain the later instar animals insufficient quantities, both O'Farrell and Stock and Bodenstein resorted to raising mass colonies of cockroaches and separating groups of animals which molted on a given day and using these selected groups for experiments.

At the time these studies were begun, I did not have the time, facilities or technical help to maintain mass colonies of cockroaches in order to glean small groups of animals that were sufficiently synchronous for experiment. Therefore I set out to develop simple techniques for rearing useably synchronous colonies of the German cockroach *Blattella germanica* and the American cockroach, *Periplaneta americana*.

In early attempts to produce synchronous colonies, I started out with nymphs which hatched on a given day. These were fed in an incubator at 29ºC and at each molt the nymphs were collected as they finished molting and stored in a cold-room without food (16ºC) until all the nymphs had molted. They were then replaced in the incubator, allowed to feed and undergo another molting cycle. By chilling the earlier animals to ecdyse at each molt until the last animal had finished ecdysis, I hoped to curtail the progressive asynchrony which crept into the culture. The method worked remarkably well. The chilling was quite unnecessary, however, since it was subsequently discovered that starvation was sufficient to prevent the earlier molting animals from getting a head start in development. This early object lesson was a caution to me in future experiments to keep methods simple and well defined, looking out for errors in experimental logic.

II. MATERIALS AND METHODS

A. Description of the Animals Used

The cockroach is a primitive pterygote. insect having a common origin with the termites from the earliest winged insect stock (McKittrick, 1965). It is a paurometabolous insect, i.e. the larvae resemble the adults in general body form, occupy the same habitat and eat the same foods. Slight allometric changes in form occur during larval life such as development of wing buds. In *Blattella*, the external genitalia change slightly from instar to instar and allow identification of the sexes. (Ross and Cochran, 1960). The work described in this paper was performed on the cosmopolitan species, *Blattella germanica*, The German cockroach, and *Periplaneta americana*, the American cockroach.

Blattella germanica,(L.) (Blattellidae) is a small species weighing about 60 mg. as unfed adults. (Fig. 1). There are normally 6 larval instars. The primary reproductive cycle in the female has been studied by Roth and Stay (1964). The female lays 30-40 eggs per reproductive cycle, encloses them in an ootheca and carries them protruding from the tip of the abdomen until hatching. Each reproductive

cycle takes about 25 days from hatching to hatching. I have used three basic strains of *Blattella germanica*. A pen inbred strain, New York (NY), was established from a gravid female from New York City. Much of my early work was performed exclusively on the strain NY. I obtained two pen inbred strains of *B. germanica* from Dr. Mary Ross of Virginia Polytechnic Institute. One strain is Orlando (ORL) named for its earliest known origin in a laboratory in Orlando, Florida . (Ross and Cochran, 1965, 1966).

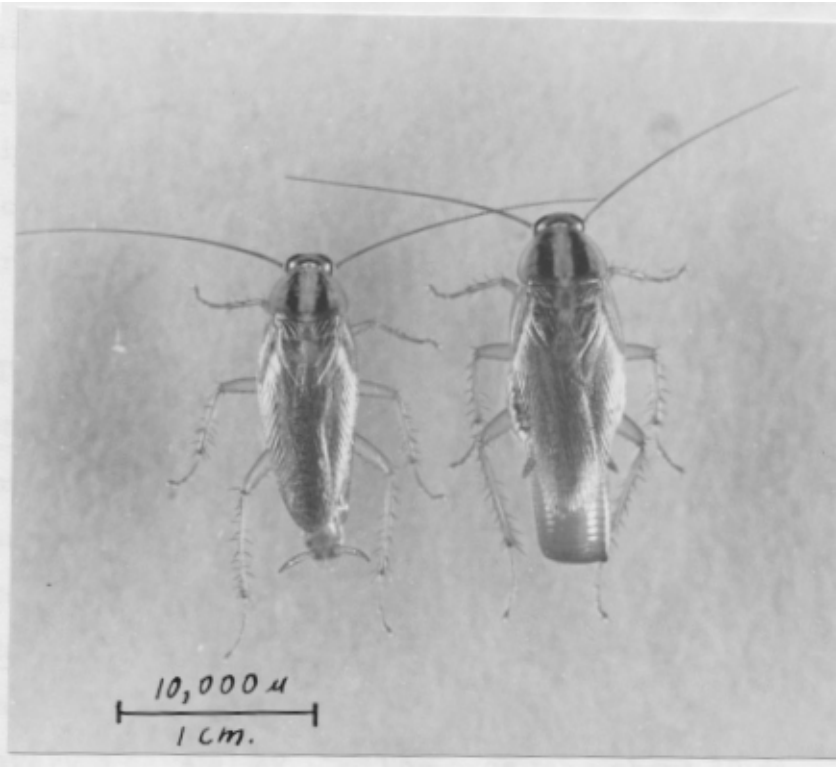


Fig. 1. *Blattella germanica* (L.) adult male (l.) and adult female (r.) with an ootheca. (~6 times natural size)

This strain carries a homozygous recessive allele called orange body (or). The larvae and adults homozygous for or have an orange-brownish pigmentation instead of the wild type dark brown to black. The orange pigmentation is light enough to allow microscopic examination of the epidermis directly through the cuticle. This permits direct observation of the epidermis in whole mounts without resorting to sectioning. The other strain of *B. germanica* I have used is Chlordane Resistant (CR) which originated in Corpus Christi, Texas. This strain carries an allele, broad-banded pronotum (bbp) which causes increased pigmentation in the cuticle. This strain was used mainly for comparison with the other strain ORL obtained from Dr. Ross.

The other species of cockroach which I used extensively is *Periplaneta americana* (L.) (Blattidae). This species was useful when large animals were needed for experiments; the adults weight about 800mg. and there are normally 9 larval instars. The female lays eggs in batches of about 16. These are

packaged in an ootheca similar to *Blattella*'s, but the female deposits the ootheca instead of carrying it about until the eggs hatch. The female can produce an ootheca of this sort every 3 days at 29ºC. The eggs take about 30 days to hatch. The strain of *Periplaneta* I used was derived from oothecae collected from stock colonies maintained by Dr. E. S. Hodgson of Columbia University, New York.

B. Techniques of Culturing

All cultures of roaches are maintained in relatively synchronous development by controlling food availability. This drastically reduced the amount of time expended in order to produce a given number of experimental animals of a given age on a given date. The experimental basis of this method will be handled in the result section but its value is such (it provides healthy larvae of a relatively homogeneous age) that the detailed culture procedure will be described here.

The animals are raised in glass finger bowls. Large finger bowls (20 x 10 cm.) could comfortably hold up to 1000 first instar nymphs, 500 fourth instar nymphs, or 30 8th-10th instar *Periplaneta* without resulting in crowding effects. A light rim of petrolatum prevents the roaches from escaping from the bowl. Water is constantly available from vials corked with a tissue wick. Purina Rat Chow is used as a complete diet, un-supplemented. The animals are handled with a water suction operated aspirator which eliminates autotomy of limbs and other injuries usually inflicted when the animals are handled manually. Before ecdysis begins, food is removed from the culture dish. As animals in a culture begin to undergo ecdysis, the newly-molted animals are periodically transferred using the aspirator to a new bowl until all the animals are molted. Unless this is done, the first nymphs to finish molting may become hungry and cannibalize some of the later nymphs when they are defenseless while wriggling from their old skin. This habit of cannibalism is a contributing cause why non-synchronous mass colonies become more asynchronous and contain many injured, unhealthy animals. The *Blattella* adults are synchronously pen mated in groups of 50 to 100 of each sex in large finger bowls. Conveniently, the oothecae carried by *Blattella* females develop a green longitudinal line on them 8 days before hatching (Clayton, 1959). Females carrying such ripe oothecae are placed in half-ounce creamers until the oothecae hatch. The hatched nymphs from a number of females are pooled to start a new synchronous colony and the females are returned to a new breeding bowl to undergo another reproductive cycle. Three synchronous reproductive cycles are obtained from each batch of females by controlling their feeding as in the nymphal development; after this, reproduction falls off sharply and they are therefore discarded. Because all the critical events which need attention in a culture such as feeding, molting, breeding and hatching are synchronous, the culture must be attended only at these critical times.

Periplaneta colonies are maintained in a similar way except that since females do not carry the egg case until they hatch, oothecae are collected periodically from a mass adult breeding culture and incubated separately until the nymphs hatch.

Experimental breeding cultures were maintained in a constantly lit room or incubators to eliminate synchronizing any light-entrained circadian rhythms. Humidity was not monitored but was maintained high by keeping a bowl of water in the bottom of the incubator.

C. Experimental Techniques

Whole mounts of the epidermis of *Periplaneta* and the homozygous mutant orange body of *Blattella* were suitable for light microscope examination, Bouin's fixative was found the most suitable for

nuclear density and mitotic studies.

Preparation of tissue for the electron microscope was carried out according to the method of Locke (1966).

D. The Statistical Treatment of Data

The usefulness of statistics lies in its ability to transform problems of the real world into abstract problems in mathematics. If the transformation is valid, a solution of the numerical problem will also solve the real word problem. It is therefore convenient when possible to design problems which are easily solved when transformed to mathematical notation. I have used, whenever possible, standard experimental designs and methods of data analysis. These methods can be found in a number of texts which I have consulted freely ...

- linear and multivariate linear regression (Mood and Graybill, 1963; Cochran and Cox, 1950; Scheffe, 1959; Dixon and Massey, 1957)

- factorial design (Mood and Graybill 1963)

- contingency (Mood and Graybill 1963; Siegel 1956)

- probit analysis (Kinney 1952)

Randomization of animals into treatment groups was performed using random number tables or drawing lots.

III. RESULTS

A. The Role of Feeding in the Control of Molting and Development

In search of methods of increasing and maintaining synchrony in cultures of cockroaches, feeding was a natural variable to investigate. Feeding has been implicated in the control of molting of a number of insects (Edwards 1967). The bed bug *Cimex* and the blood-sucking bug, *Rhodnius* (Buxton 1930; Wigglesworth 1934) are intermittent feeders and a blood-meal in either will initiate a molting cycle. *Tineola*, however, a pest of stored clothes, undergoes a frenzy of molting when starved, decreasing its size with each molt that it undergoes in the absence of food. Therefore there was *a priori* evidence for both alternatives, that in the cockroach feeding would either he helpful in controlling the molting cycle or that molting cycles would continue even in the absence of food, depending upon whether the cockroach was analogous to the intermittent feeders, *Rhodnius* and *Cimex*, or to the clothes moth, *Tineola*.

In order to test whether feeding has a role in controlling molting, various experiments were performed on the strain New York (NY) with food availability as a variable. Colonies of cockroaches in which food is constantly available soon become markedly asynchronous (Fig. 2). If the molting cycle were dependent on some extrinsic factor such as food for initiation, it should be possible to eliminate the additive factor in the increasing asynchrony of molting by controlling the availability of food.

Feeding experiment #1

In a pilot experiment a control colony of 35 larvae from a single ootheca was raised at 29ºC with food constantly available (Fig. 2-O). Three other colonies were raised under similar conditions, but food was removed before the colonies started molting and reinstated only after all the larvae had molted (Fig. 2-A,B,C). Progressive asynchrony was eliminated in colonies in which the animals began feeding simultaneously in each instar.

The adults derived from colonies A, B, and C were interbred at 26ºC and oothecae were produced relatively synchronously. After the eggs hatched, the females were again fed simultaneously and allowed to mate with the males, and again a synchronous batch of oothecae was produced (not shown in Fig. 2). By controlling the availability of food to the adult females only, synchronous ootheca production and hatching can be repeated up to 3 or 4 times. Mating is not necessary during every reproductive cycle, but the maximum number of larvae per ootheca was obtained when males were available. A synchronous breeding colony of 30 females and 30 males can thus produce 900-1200 synchronously hatched larvae at regular intervals defined by the feeding schedule of the females.

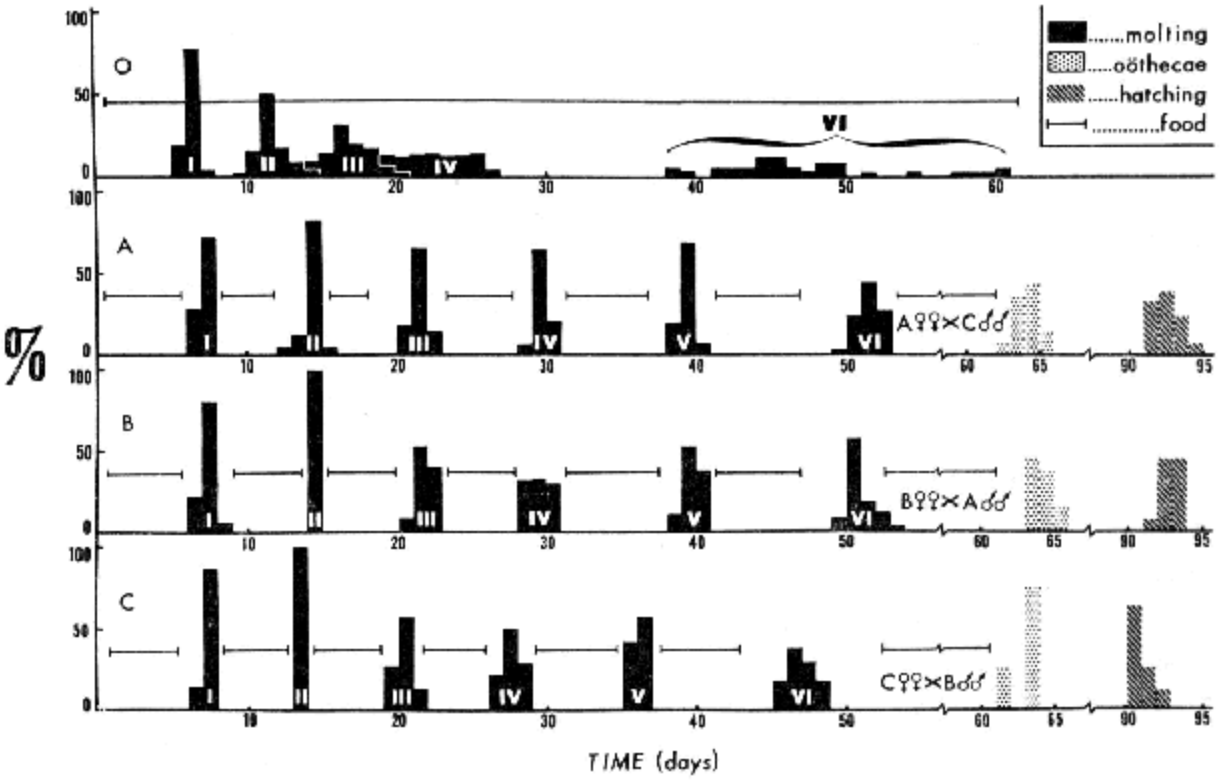


Fig. 2. The effect of controlled food availability on the synchrony of molting in colonies of the German cockroach, *Blattella germanica*. Each colony (A, B, C, and O) of 35 to 40 larvae hatched from a single ootheca. Adults of colonies A, B, and C were outbred as indicated for maximum viability. The production and subsequent hatching of oothecae are indicated for the first ovipositional period only.

Feeding experiment #2

To substantiate further the availability of food as an extrinsic factor in the control of the molting cycle, five sub-colonies (D, E, F, G, and X, Fig. 3) of 50 unfed fourth instar larvae were isolated from a larger colony of larvae which all molted on the same day. Sub-colonies D, E, F, and G were placed in a 30ºC incubator and given food 1, 3, 5, and 7 days respectively after they had molted to the fourth instar. Sub-colony X was similarly treated but not given any food and did not molt. Figure 3 shows the direct dependence of the time of molting on the availability of food.

Feeding experiment #3

Since initiation and maintenance of the molting cycle could be associated with either the beginning, the entire length, or the termination of feeding, an experiment was designed to determine which phase of the feeding period was related to the initiation of molting. Five colonies of 50 larvae each were isolated from a larger well-fed colony as they molted to the fourth instar.

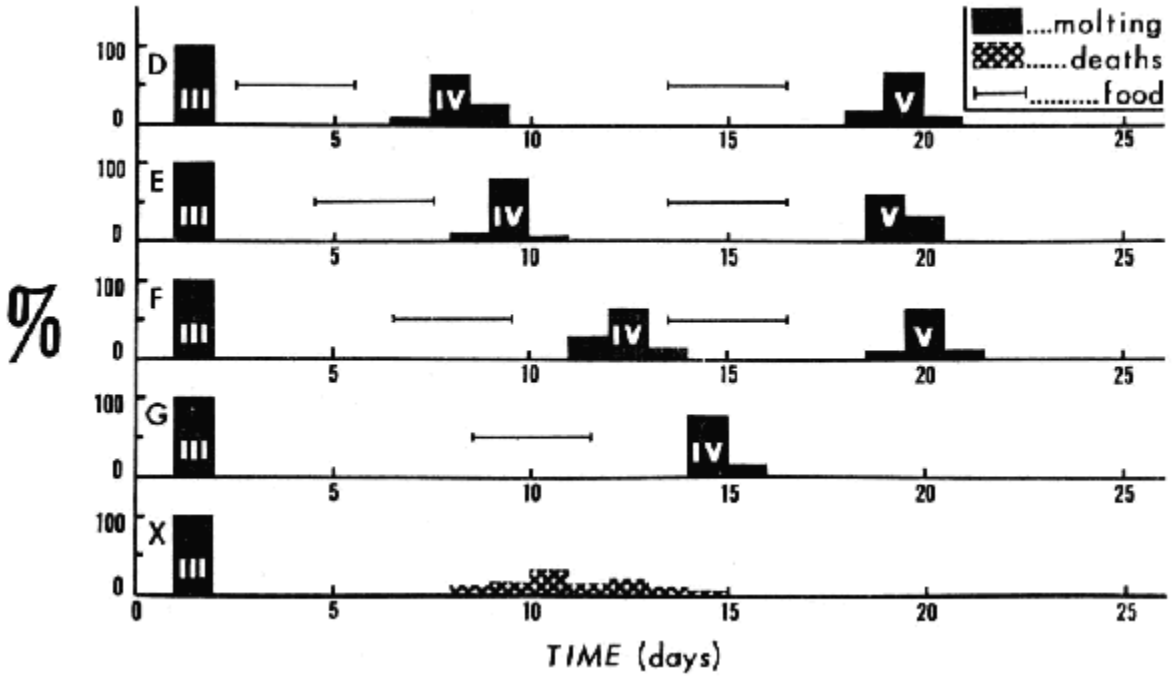


Fig. 3. Dependence of molting on the time of food availability in the German cockroach, *Blattella germanica.* Each colony of 50 larvae was isolated at the third molt from a larger colony kept synchronous as in Fig.2 A,B,C.

These colonies of larvae were starved for a day at 30ºC and then were all fed at the same time. Food was removed 0.5, 1.0, 1.5, 2.0, and 2.5 days respectively after it had been presented. Ninety percent of the larvae fed for only 1 half-day molted to the succeeding instar. Thus 1 half-day of food availability was sufficient to initiate a molting cycle in these cockroaches. All larvae fed for 1 day or more molted to the next instar. All the larvae which did molt underwent ecdysis at the same time, 5 days after the food was presented. This indicates that the initiation of the molting cycle in cockroaches is associated with the early phase of feeding and is not dependent on sustained feeding.

Feeding experiment #4

The fact that 90 per cent 'of the animals fed for a half day in the fourth instar molt to the fifth instar does not assure that 12 hours of feeding per instar is sufficient to sustain growth and development from the egg to the adult. Accordingly an experiment was designed to test the effectiveness of various lengths of feeding in sustaining growth and development. Four lengths of food availability per molting cycle were used (24, 48, 76, 96 hours}. From a large population of newly hatched first instar nymphs, individuals were randomly assigned to 4 bowls, 60 nymphs per bowl. Drinking water was available to the animals throughout the experiment. Lengths of feeding were assigned randomly to the 4 bowls. Food was made available to the animals in the 4 groups for the assigned times. A record was kept of the number of nymphs which reach each instar of development. Table 1 gives the percentages of each treatment culture which reached each instar :

Table 1. Food availability on percentage reaching each instar.

Hours of food/instar	I	II	III	IV	V	VI
24	100	20	-	-	-	-
48	100	91	44	34	19	-
72	100	100	100	100	100	100
96	100	100	100	100	100	100

This data indicates that between 48 and 72 hours of feeding time per instar (120-140 hours) is necessary to sustain growth and development.

The contrast between the results of feeding experiments #3 and #4 is interesting. According to experiment #3, a nymph fed ad lib in the previous instar can complete another molting cycle with only 12 hours of feeding while experiment #4 demonstrates that an animal cannot sustain growth and development on 48 hours of feeding in every instar. This is a strong indication that all food taken in is not used for immediate tissue growth and that considerable storage of food occurs between instars if food is available for more than 48 hours per instar.

Clearance figures are available for the rate at which the gut empties after a meal in a number of species of insects including the cockroaches *Blattella germanica* and *Periplaneta americana* (House, 1964). The speed at which food moves through the alimentary canal (empty 4 hours after feeding stopped) suggests that the food present in the crop at a given time is a good indication of the amount of recent feeding. For this reason an experiment was designed to measure quantitative changes in gross fresh weight (GFW), crop fresh weight (CFW), and residual fresh weight (RFW = GFW-CFW) during the cockroach molting cycle.

A colony of 400 fourth instar *Blattella* nymphs were starved for 4 days after their previous ecdysis, food was made continuously available and samples of 20 animals were taken at 0, 12, 24, and 120 hours after feeding. The gross fresh weight of each animal was taken, the animals dissected and the crop fresh weight of each animal was measured. The results of this experiment are shown in Figure 4 as the sample mean (+/- a 95 per cent CI of the mean) of each weight component.

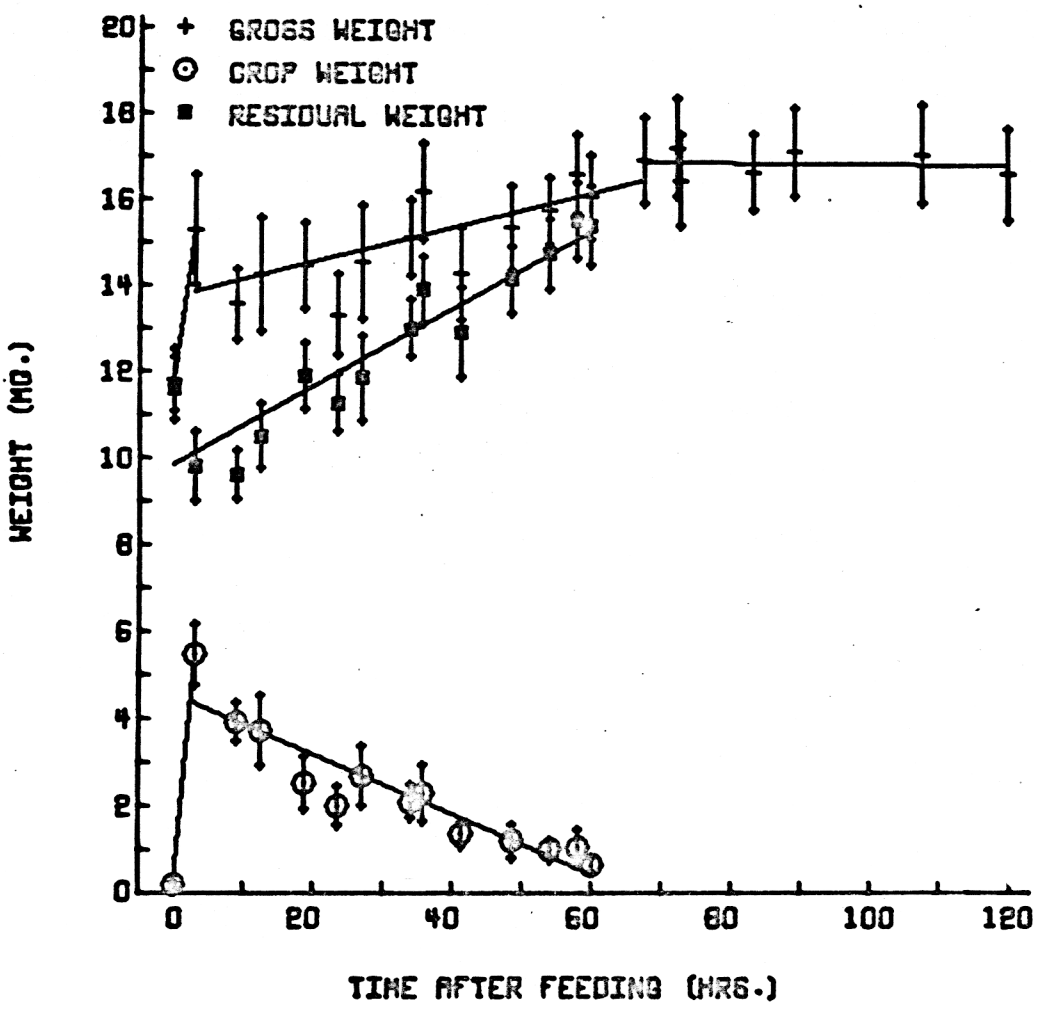


Fig. 4. Weight changes during the IV instar of *Blattella gerrnanica* (L.). Samples of 20 animals were taken from a large culture synchronized by feeding at various times after food was made available. The components of weight followed were gross fresh weight (GFW), crop fresh weight (CFW), and residual fresh weight (RFW). RFW = GFW - CFW . Plotted are the sample means of each component with a 95% confidence interval of the mean.

The main points of interest of the curves are the leveling off and converging of gross fresh weight and residual weight at about 72 hours, the very rapid weight gain of the crop soon after food becomes available, and the virtual emptiness of the crop towards 72 hours after food becomes available. The ability of the cockroach to molt after a brief feeding may be due to its ability to fill its crop maximally in a short time. This is of particular use to a scavenger which must make quick use of the food it comes upon. If food clearance is as rapid as indicated in the literature (4 hours), this curve demonstrates that feeding still occurs after 48 hours although it falls off to virtually nothing by 72 hours. The feeding process is probably more complicated than I assume but this does not drastically alter my conclusions.

To summarize these experiments on feeding, it can be said that the cockroach *Blattella* is similar to the intermittent feeders *Rhodnius* and *Cimex* in that the initiation of a molting cycle is dependent on feeding. However as is shown in contrasting experiments #3 and #4, the amount of feeding which it

takes to maintain a molting cycle depends upon the amount of feeding which occurred in previous instars as well as the current one.

Some practical knowledge is derived from this experimental section. First, controlling feeding is a convenient method of maintaining synchronous colonies of cockroaches. The synchrony of a culture of cockroaches makes it easier to maintain a relatively homogeneous source of experimental animals and also simplifies the breeding techniques necessary to provide cultures of synchronously hatching nymphs for establishing new synchronous cultures.

Also the amount of feeding time available to the cockroach was found to be critical in the control of molting. If experiments are to be performed which impair feeding, then care must be taken that the animals had sufficient food to continue development in the course of the experiment. If the animals have been well fed in the previous instars, then 24 hours of feeding in the fourth instar is enough to insure initiation and maintenance of the molting cycle.

B. Factors Affecting the Molting Cycle

In any study of growth and development it is always necessary to keep in mind the effects of factors extraneous to the major aims of the experiments being performed. The following section deals with some of these extraneous factors which I have found necessary to take into account in my design of experiments.

1. Temperature

The gross response of growth rate of *Blattella* to temperature has been published in various sources. I have compiled some of these sources into Figure 5. Since the methods of measuring the rate varied considerably from author to author, the variance about the regression line fit to the data is not too meaningful. The maximal rate of growth reported for *Blattella* occurs at 30ºC. Above 30ºC in the cockroach the water permeability barrier in the cuticle breaks down (Beament 1964) and the animal dehydrates rapidly in dry air.

A choice of temperature for performing experiments therefore involves two opposing factors, the increased rate of development afforded by temperatures close to 30ºC and the detrimental effects of temperatures above 30ºC.

The concept of upper lethal limit as given in texts (Wigglesworth et al. , 1964) is of little use when applied to this problem. Therefore a few pragmatic experiments were performed to determine what the effect of short and long term fluctuations of temperature above 30ºC have on the molting cycle and development of the German cockroach, *Blattella germanica*.

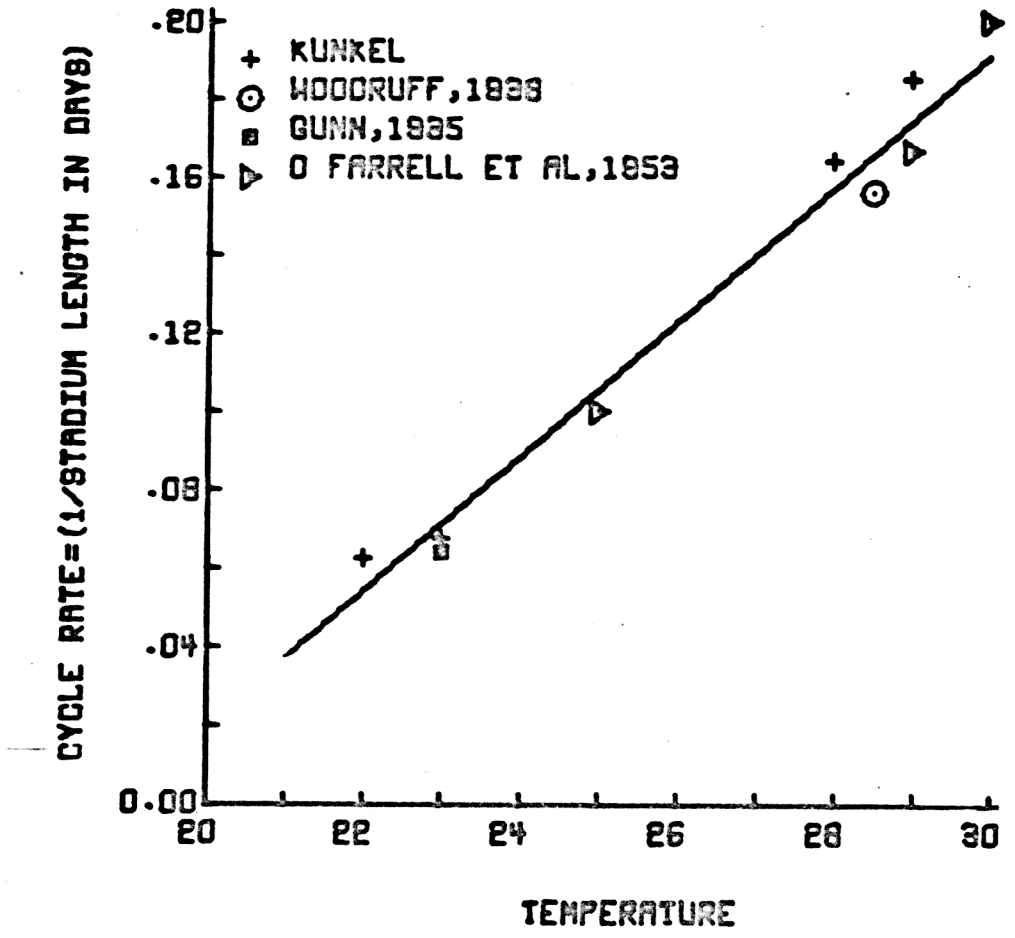


Fig. 5. Compilation of molting cycle rates of *Blattella germanica* measured by various authors.

When I attempted to grow *Blattella* at a constant temperature of 37ºC in a saturated atmosphere, to prevent water loss, the animals lived up to 7 days but invariably died without molting. At lower constant temperatures of 31 to 34ºC, the animals survive and molt but the instar length is considerably longer than for 30ºC. When sixth instar animals, which would normally metamorphose to an adult at the next molt were raised at these temperatures (31-34ºC), metamorphosis is prevented producing "giant" supernumerary seventh instar nymphs. These nymphs are different from the spontaneously occurring supernumeraries reported by O'Farrell and Stock in that they are significantly heavier than normal pre-imaginal nymphs and if subsequently allowed to metamorphose to eighth instar adults, these adults are 20 percent heavier than their seventh instar adult controls. Thus although growth still occurs at these higher temperatures, the hormonal signals for metamorphosis or the tissue's response to the signals is interfered with. A further experiment showed that even a short fluctuation of one hour at a higher temperature of 37ºC early in the sixth stadium would induce this inhibition of metamorphosis (cf. Okasha, 1964).

In order to prevent my experiments from turning into a study of artifacts of temperature

fluctuations, I decided to perform all my experiments at 29ºC allowing for the one degree fluctuation in temperature inherent in my incubators. This temperature gave rapid and consistent growth with a low incidence of supernumerary nymphs.

2. Incubator effects

Although the incubators I used, maintained a regulated temperature to ±1ºC, they had their heating elements at the bottom which heated by convection and thus did not give a uniform growth rate throughout. If experiments on growth rate are run with animals dispersed in a number of bowls, the position of the bowl could be confounded with the experimental treatment. An experiment was designed to test how serious the position effect was in the incubator.

Sixteen bowls of randomly selected unfed fourth instar ORL strain animals were arranged in 4 rows and 4 columns, about 100 animals to a bowl. The animals and bowls were allowed a half day to come to the temperature in their position in the incubator. They were fed simultaneously and the time they underwent ecdysis was recorded. Table 2 gives the analysis of the data.

The analysis shows that both row and column effects are significant. The partitioning of the row effect shows that there are highly significant linear and quadratic components of the row effect; the animals in the bottom-most row develop the fastest
while the top-most row develops the slowest. The mean linear difference between two rows is l.86 ± 0.54 hours. This is a small difference considering the mean length of the instar is 148.5 ± 0.5 hours. Since the columns of bowls were not arranged in any particular linear array, the column effect was not partitioned. These gradients are most likely a result of the incubator's heating element being in the bottom of the incubator, producing slightly different temperature conditions in the stacked bowls. In any event, these gradients of growth rate must be taken into consideration when planning an experiment in which the animals are distributed in 2 or more bowls. It is of particular interest that there is no significant ($p > 0.1$) row-column interaction because in many standard experimental designs such as Latin Squares and Graeco-Latin Squares (Cochran and Cox, 1950) the treatment effects would be partially confounded with the row-column interaction by the design of the experiment itself. If no row-column interaction exists, the treatment effects can be trusted.

3. Strain differences

Although my early work on *Blattella germanica* was exclusively restricted to a pen inbred NY, I wanted to incorporate also the ORL and CR strains into my research. I performed a test to compare their molting cycle lengths. Since strain CR could be superficially confused with strain NY, the strains were raised in separate bowls and arranged in a 3 by 3 Latin Square so that row and column effects could be controlled. Table 3 is the analysis of variance of the data obtained. The difference in cycle length due to strain is highly significant. The mean molting times for NY, CR and ORL are respectively 128.7, 127.7, and 114.3 hours. In addition a Bartlet's test of homogeneity of variance (Dixon and Massey, 1957) shows that the variability of ecdysis is not the same for the nine cultures ($P < 0.001$). Table 4 gives the variances of molting for the nine bowls. It is obvious that the strain Orlando has a smaller variance of molting time than the other two strains. This inequality of variance sheds some doubt on the estimation of differences performed in Table 3, since the method of estimation assumes equal variance for the different treatment groups. However the method is known to be robust or insensitive to deviations from this assumption when large samples and equal sample sized treatments are used {Scheffe, 1959). Other experiments in this thesis also reap this reward for using large and equal sample size. In short, there is a good deal of variability in developmental rate between different

strains of *Blattella germanica* and care must be taken in comparing the data taken from the different strains.

TABLE 2.--Analysis of incubator gradients affecting growth rate in the fourth instar larvae of Blattella germanica

Equation to be fit: $Y_{ijk} = U + R_i + K_j + R_iK_j + e_{ijk}$

Y_{ijk} = time from feeding to ecdysis
U = grand mean of ecdysis times
R_i = effect of row i ; i= 1, 2, 3, 4
K_j = effect of column j ; j= 1, 2, 3, 4
R_iK_j = interaction between row i, column j
e_{ijk} = error associated with ijk^{th} animal

ANOVA

Source	D. F.	Sums of squares	Mean square	F Ratio
Row Effect (R_i)	3	7,786.8	2,595.6	40.38**
Linear	1	7,277.7	7,277.7	113.**
Quadratic	1	462.0	461.9	7.19**
Cubic	1	47.1	47.1	.7
Column Effect(K_j)	3	10,096.0	3,365.3	52.35**
Row-Column interaction	9	952.4	105.8	1.64
Residual	1664	106,952.8	64.2	
Total	1679	125,788.0		

** significant at .01 level

TABLE 3.--Analysis of variance of molting cycle length at 29°C due to three different strains- New York (NY), Orlando (ORL), and Chlordane Resistant(CR)

Equation to be fit: $Y_{ijk} = U + R_i + K_j + S_k + e_{ijkl}$

Y_{ijk} = time from feeding to ecdysis for ijk^{th} animal
U = a grand mean of ecdysis times
R_i = effect of row i ; i=1, 2, 3
K_j = effect of column j ; j=1, 2, 3
S_k = effect of strain k ; k= 1, 2, 3 =NY, ORL, CR
e_{ijkl} = error associated with $ijkl^{th}$ animal

ANOVA

Source	D. F.	Sums of squares	Mean square	F ratio
Strains	2	39,924.25	19,962.12	147.4**
Row effects	2	11,893.26	5,946.89	43.9**
Column effects	2	3,989.26	1,994.63	14.73**
Residual	924	125,084.23	135.37	
Total	930	180,891.54		

**significant at .01 level

Table 4. – Comparision of variability of molting cycle length within strains of the German cockroach, *Blattella germanica*

Strain	Replicate	Variance of Molting time	Pooled variance
New York (NY)	1	202	
	2	183	175
	3	147	
Orlando (ORL)	1	73	
	2	92	80
	3	77	
Chlordane Resistant (CR)	1	175	
	2	98	133.5
	3	130	

One of the primary aims in obtaining a strain with an easily recognizable genetic marker was the possibility of performing an experiment in which both experimental and control animals could be raised together in the same bowl and be easily recognized. The gene *or* is a recessive trait in which the cuticle of the animal is orange and easily distinguishable from wild type coloration. The strain ORL has the *or* gene as well as having a shorter molting cycle and higher synchrony than other strains. All that was necessary to make the ORL strain ideal for experiment would be to introduce the wild type coloration gene *or*+. This would allow colonies to be raised which were half *or*+/*or*-, phenotypically wild type, and half *or*-/*or*-, phenotypically orange body. In order to achieve this, the NY strain was crossed with the ORL strain and then heterozygotes, *or*+/*or*-, subsequently back-crossed with ORL strain in a standard program to dilute out the NY chromosomes and genes.

Blattella germanica has 11 autosomals plus 1 or 2 sex chromosomes (male XO, female XX) (Stevens, 1905; Wassilief, 1907). Some resistance to dilution of NY genes linked to *or* might be met if crossover of the chromosome bearing *or* is low (cf. Ross and Cochran, 1967). At each dilution the difference in molting time between *or*+/- and *or*-/- individuals was estimated to approximate the dilution resistance (Tables 5, 6). Table 7 is a summary of the results of comparison of the phenotypes *or*- and *or*+.

TABLE 5.--Estimation of the mean difference of molting cycle lengths associated with phenotypic differences in the F_2 generation of the hybrid strain ONY (1:1)

Equation to be fit: $Y_{ijk} = U + B_i + P_j + e_{ijk}$; i=1,2 j=1, 2 k= 1, 646

Y_{ijk} = time from feeding to ecdysis in IV instar of ijk^{th} animal
U = mean time of molting
B_i = effect of bowl i ; i= 1, 2
P_j = effect of phenotype j; j= 1, 2;1=or^-, 2=or^+
e_{ijk} = error associated with ijk^{th} animal

ANOVA

Source	D. F.	Sums of squares	Mean square	F ratio
Bowl effect (B_i)	1	664.19	664.19	4.97*
Phenotype effect (P_j)	1	2,265.28	2,265.28	16.95**
Residual	643	85,927.73	133.64	
Total	645	88,857.20		

*significant at .05 level

**significant at .01 level

Mean difference in molting cycle length of or+ and or- = $P_{or+} - P_{or-}$ = 3.75 ± 1.26 hrs.†

†95% confidence interval of the mean

TABLE 6.--Estimation of the difference of molting cycle lengths associated with phenotypic differences in the hybrid strain ONY (1:3)

Equation to be fit: $Y_{ijk} = U + B_i + P_j + e_{ijk}$

Y_{ijk} = time from feeding to ecdysis in III instar of ijk^{th} animal
U = mean time of molting
B_i = effect of bowl i, i= 1, 2
P_j = effect of phenotype j; j=1, 2 ; 1=or^- , 2=or^+
e_{ijk} = error associated with ijk^{th} animal

ANOVA

Source	D. F.	Sums of squares	Mean square	F ratio
Bowl effect (B_i)	1	1,033.47	1,033.47	16.05**
Phenotype effect(P_j)	1	505.77	505.77	7.85**
Residual	649	41,793.14	64.40	
Total	651	43,332.38		

**significant at .01 level

Difference in mean molting cycle length of or+ and or- =

$$P_{or+} - P_{or-} = 1.44 \pm 0.87 \text{ hrs}^{\dagger}$$

†95% confidence interval of the difference

Table 7

Dilution level	Mean difference between molting time of or+ and or-
parents NY, ORL	14.43 +/- 1.32
ONY(1:1)	3.75 +/- 1.26
ONY (1:3)	1.44 +/- 0.87

Despite the fact that residual differences in molting cycle lengths still existed between the two phenotypes in the 1:3::NY:ORL diluted level, these animals could still be used in growth rate experiments as long as the treatments were applied in an experimental design which also tested and eliminated the dilution-resistance effect. Since this dilution-resistance effect is not of any particular interest in an experiment, it can be confounded with the bowl effect or some other design component which is not of prime interest.

C. Temporal Mapping of Molting Cycle Events in the Colony and the Individual

A great deal of information about development is contained in a description of the temporal sequence of events within an individual. Of course the ultimate aim is to discover the causal relationship between the events; but in order to set up experiments on these events, a knowledge of when they occur is essential. A complete description of the development of a particular individual could be given as a particular sequence of events (A, B, C, ...). However a more accurate description of the more generalized individual would be to give all the actual sequences which occur in nature, with estimates of the preferred sequences. For example given three events (A, B, C) there are 3! = 6 possible sequences in which they might occur but in nature they may actually only occur in one or two sequences of the 6, and one particular sequence may occur 90 per cent' of the time. Thus an ideal basis for experiments in development would be a description of critical developmental events, the actual time sequences in which they can occur, and the preferences or probabilities of a given sequence. For example, it is already known that regeneration, which is a series of events, must occur before an event called the regeneration critical period or reg c.p.

In trying to reach this ideal basis for experiment a number of problems arise. First, the types of events that can be studied are limited to those for which an adequate assay is available for whether the event is occurring or has occurred. Secondly, assaying for one event may make it impossible to assay for a second event in the same animal. This is a particularly serious complication because as already stated the physiological age of the animal is not known absolutely, and the results of the assays for 2 events performed on separate animals of different unknown physiological age will be confounded with the age differences of the animals. For this reason, techniques of mapping must be used which can cope with the variability in age of the experimental material.

1. A Map of the Mean Time of Events

As a first approximation to atemporal map of the individual, a map of the sequence of the events in

a large relatively synchronous culture of animals was considered. In this type of map, one would determine the time, T_{50}, when 50% of the animals in a culture have undergone an event. In preliminary work I recorded the time of ecdysis for 72 colonies of about 100 fourth instar *Blattella* nymphs. Using a chi-square test of fit, the ecdyses of 59 of the 72 colonies could not be distinguished from a normal distribution at the 0.05 level of significance. For this reason I chose a method of estimating the T_{50} of an event which assumed the event was normally distributed in time. This method of probit analysis (Finney, 1952) fits a cumulative normal curve to estimates of the increasing proportion of animals past an event in a synchronous culture of animals. Figure 6 diagrammatically explains the approach. At an early time, t, only a small group of animals have passed the event 'A'. As time and development progress the set of animals, A, in which the event has occurred increases while the complementary set, A', declines in number. In order to determine the T_{50} of the event A one samples at various times. These samples are estimates of the true proportions of animals past the event at that time. Then relying on the assumption that the events are normally distributed in time, a cumulative normal curve is fit to the data by probit analysis. This analysis gives:

1. a test of fit of the data to a normal distribution
2. an estimate of the T_{50} of the event A.
3. an estimate of the variance of the T_{50} of A.
4. a test of parallelism of regression with another event, B.
5. If the regression of A and B are parallel, a test of sequence of the T_{50}'s of A and B.

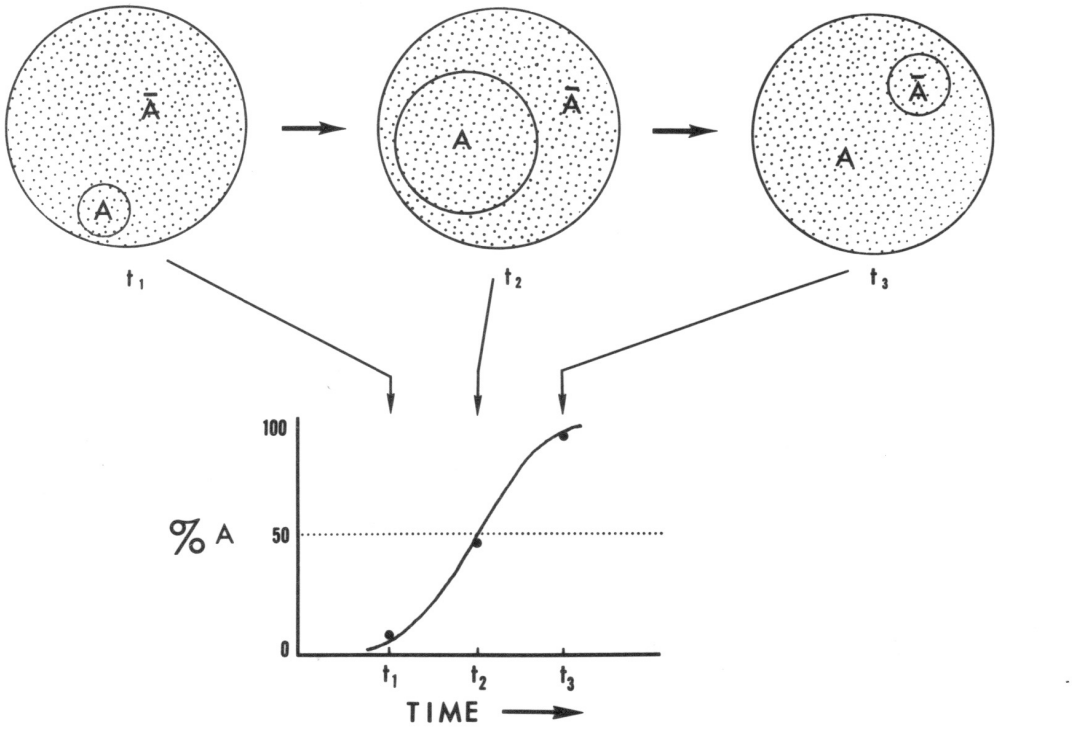


Fig. 6. The method of mapping of events A and not-A (A-bar) in time t, for a hypothetical colony of animals. At an early time t_1 the proportion of animals having undergone event A is small but grows larger with time. Random samples are taken at different times and the proportion of the sample which is A is an estimate of the true proportion. A normal curve is fit to these proportions to give an estimate of the T_{50} of the event.

This method was applied to a number of events in the molting cycle of the cockroach, *Blattella*

germanica. For the purpose of the experiments the events are best described in terms of assay of an the operational definitions used in deciding the result of an/event, while the meaning of the events is left for discussion.

Def. 1: The *critical period for the head* (head c.p.) is the time in each animal after which the head is no longer necessary to complete a molting cycle. Animals past the head c.p. will molt even through their head is ligated from their body.

Def. 2: The *critical period for the prothorax* (pth.c.p.) is the time in each animal after which the prothorax (containing the prothoracic gland) is no longer necessary to complete a molting cycle. Similar to the above assay, animals past the pth.c.p. molt even with a ligature between the pro- and meso-thorax.

Def. 3: The *critical period for leg regeneration* (reg.c.p.) is the time in each animal after which if a leg is autotomized, regeneration of the leg is postponed until the next molt (O'Farrell and Stock, 1954). Unless otherwise stated, "autotomized" will mean that I induced the animal to lose its leg by autotomy.

Def. 4: Appearance of protein granules in the fat body is indicated by appearance of Millon positive granules in fat body squashes at the resolution of the light microscope.

Def. 5: The end of epidermal proliferative mitosis is the time in each animal in which scanning of 2 entire Feulgen-stained whole-mounted tergites (10^4 cells) show no mitotic figures, given that the epidermal nuclear density is such that mitoses are known to have occurred. (Fig. 7).

Def. 6: Nerve coil appearance in the molting space is the time in each animal after which whole mounts of the epidermis show coils of nerves in what is presumably the molting space. Figure 8a shows a light micrograph of a whole mount showing the hind edge of a dorsal tergite in which a nerve coil is clearly seen. Figure 8b is an electron micrograph of a section through the molting space of the cockroach epidermis which depicts a nerve coil.

Def. 7: The beginning of bristle morphogenesis in the individual is taken as the time after which protrusions from the bristle cells are seen in the molting space in whole mounts observed in a light microscope. Figure 9 a-g are light micrographs of whole mounts of tergites showing bristles in various stages of development.

Def. 8:The end of bristle morphogenesis in the individual is taken as the time after which the new bristles of an animal have assumed their final form. Figure 9f is a light micrograph showing a new bristle folded in the molting space of a tergite.

Def. 9: The release of epidermal muscle attachments to the old cuticle is an event which occurs between the time A when the old cuticle is loose but remains attached to the new cuticle at the muscle attachments and time B when the old cuticle can be easily removed from the new cuticle. Figure 10 shows a light micrograph of a muscle attachment in which the old cuticle is attached to the new cuticle only at the muscle attachment.

Def. 10: Edcysis is a process climaxing the molting cycle which takes about 3 hours at 29ºC. It begins with the swallowing of air to inflate the animal, subsequent splitting and shedding of the old cuticle and expansion, hardening and darkening of the new cuticle. For an operational definition the event ecdysis will be taken to mean the splitting of the cuticle which is instantaneous.

Some justification must be given for considering the appearance of these nerve coils as an

important event. These nerve coils appear in the epidermis almost 60 hours before the roach is due to shed its old cuticle. In this 60 hours it is of survival value to the roach to keep in sensory contact with its environment. The production of a new cuticle however involves production of new sensory bristles extending into the molting space (Fig. 9). Since the nerve to the old bristle issues forth from the tip of the new bristle, a considerable slack of nerve is necessary in the molting fluid to allow the new bristle tip to grow to its fully extended length (Fig.9e) and still allow the nerve to reach back to innervate the old bristle . The insect provides for ample slack of nerve which is extended into the molting space as a coil. Electron microscope survey pictures show that the epicuticle shield which protects the nerve in the molting fluid extends a considerable distance through the epidermis and is most likely the source of the slack nerve plus shield needed when molting begins (Fig. 8c, d) (Slifer ,1961; Locke, 1966).

It may be noticed that although the end of epidermal mitoses is defined, no definition for the beginning of mitosis is given, this is for two reasons: First, mitoses start to appear in a part of the epidermis which is difficult to observe accurately in whole mounts, and second, the beginning of the appearance of mitoses in the epidermis is slow and ill-defined in the individual, while the end of mitoses is abrupt and more easily assayed for. However, the beginning of proliferative mitoses in the epidermis can be determined experimentally rather than by simple observation.

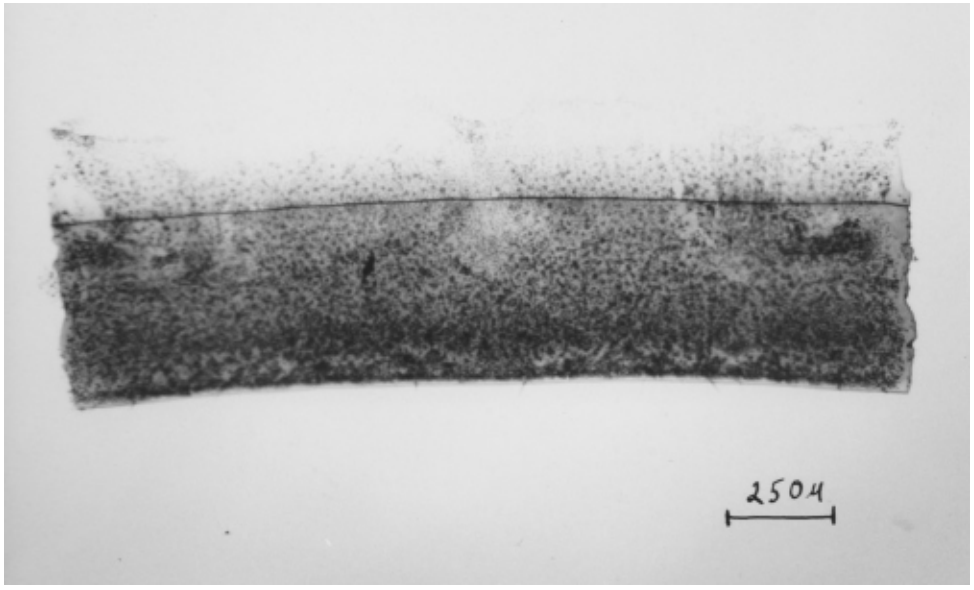


Fig. 7. Entire dorsal tergite of *Blattella germanica*. The nuclei were stained with Feulgen. There are approximately 10^4 epidermal cells per two tergites.

a)

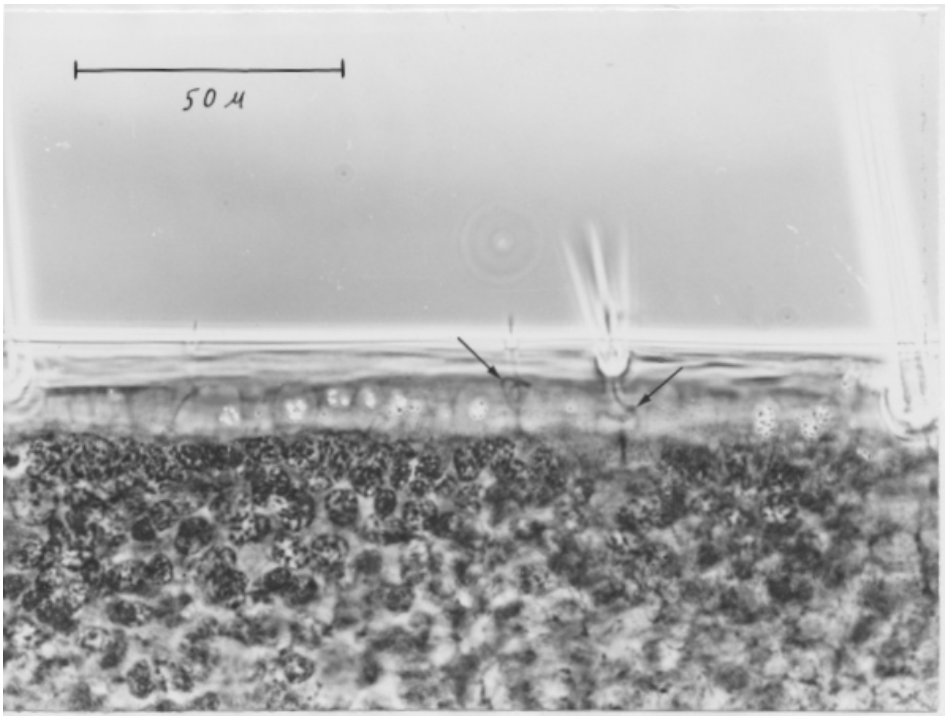


Fig. 8. Nerve coils in the molting space of the hind edge of a dorsal tergite.
a) A whole mount under phase contrast showing the nerve coils (arrows) inbetween the epidermis and the old cuticle.

b)

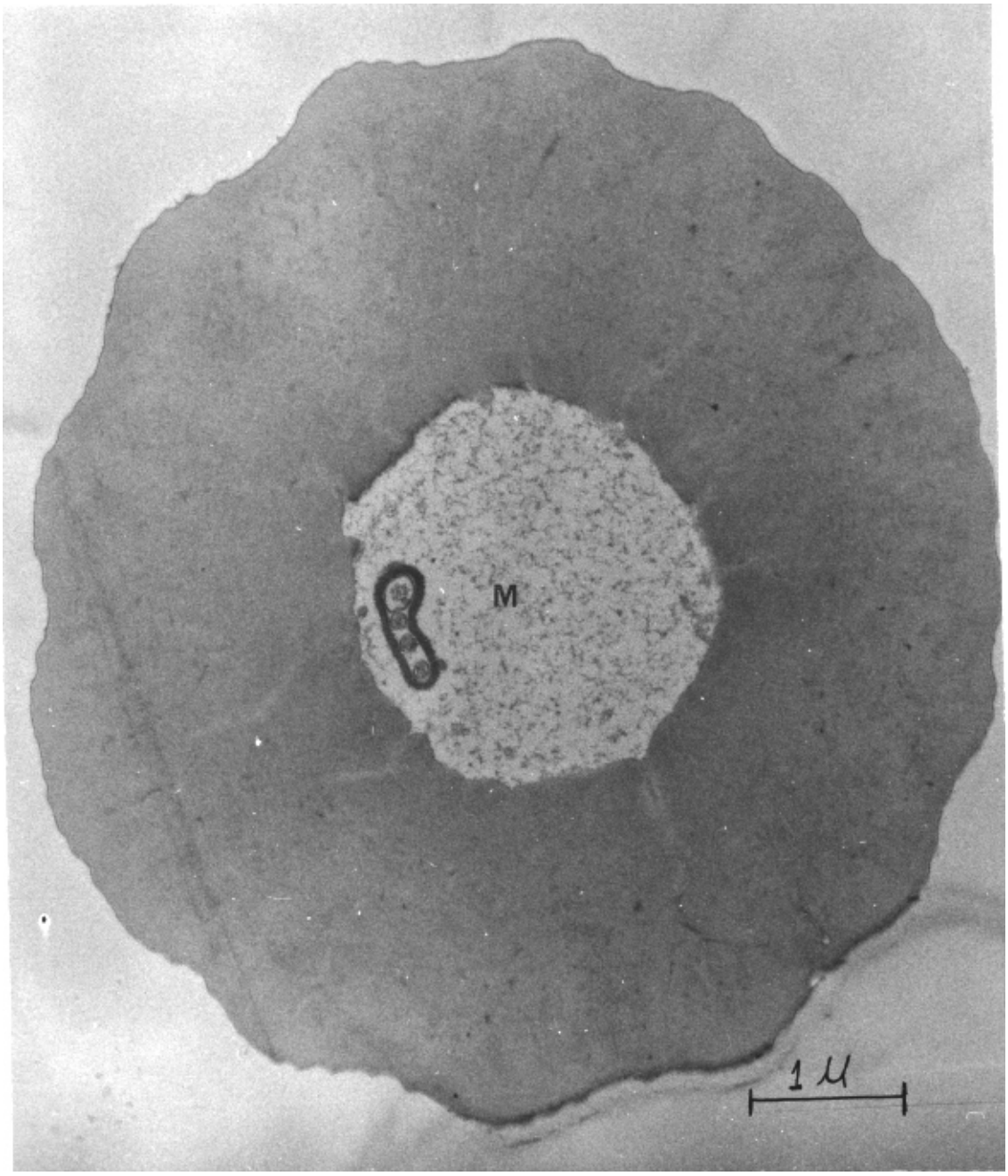


Fig 8 cntd.,

b) An electron microscope transverse section through a bristle of the cercus showing 4 nerve fibers within a protective sheath within the molting space (m). The cytoplasm of the trichogen cell has receded.

c)

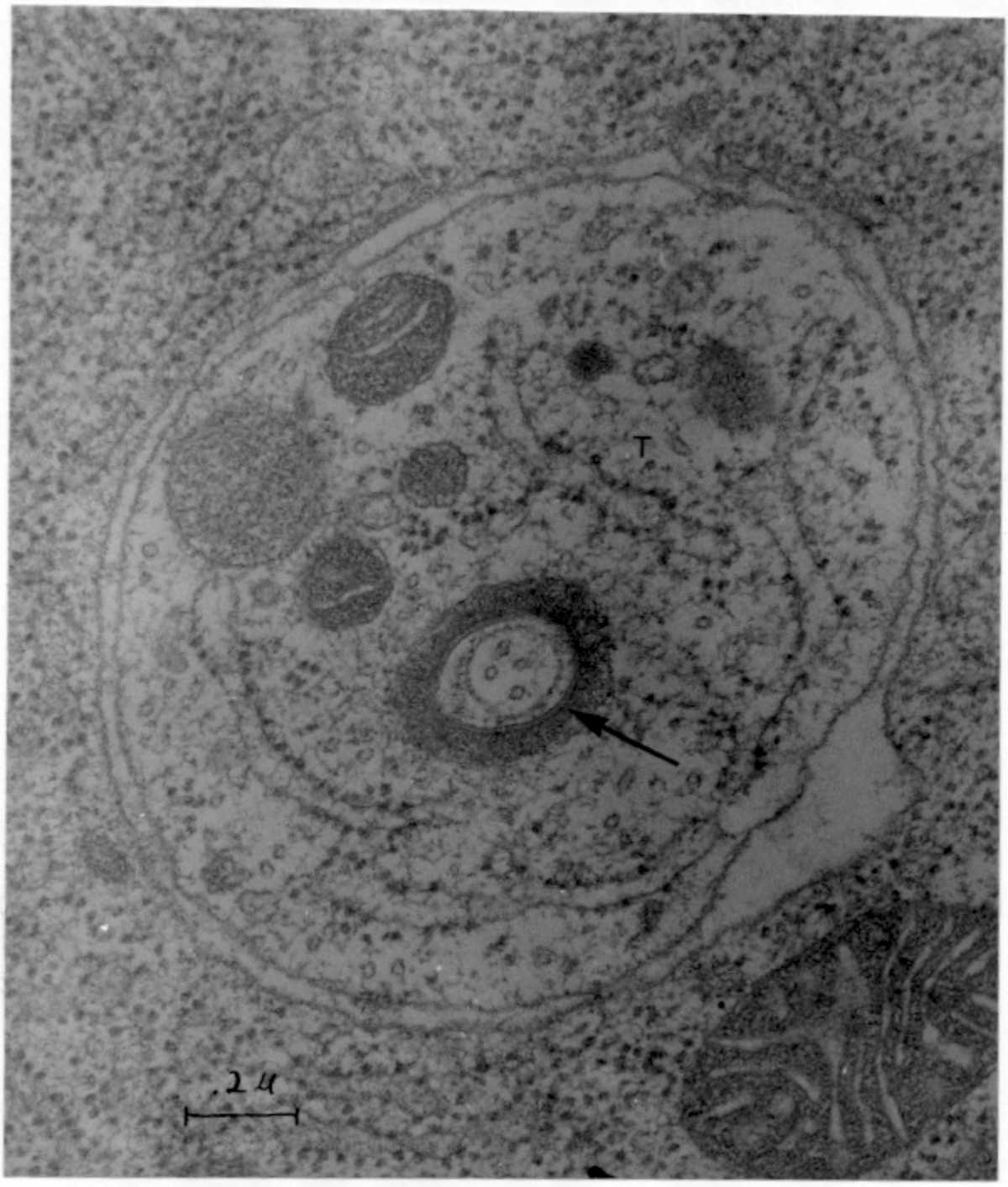


Fig 8 cntd.,

c) A nerve deep within the epidermis going to a bristle. The trichogen cell (t) which encloses the nerve is seen to have an epicuticular lined canal (arrow) in which the nerve is contained.

d)

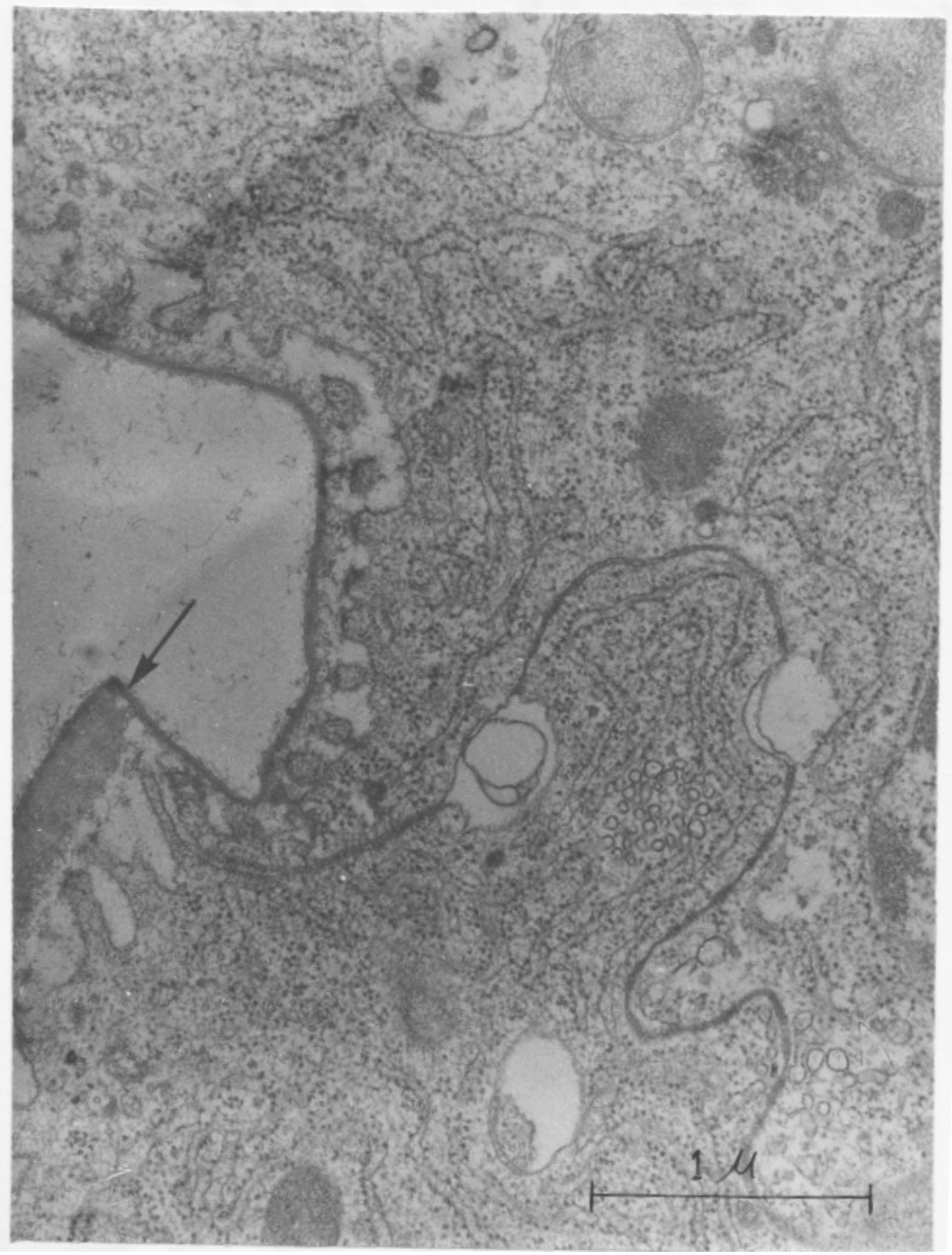


Fig 8 cntd.,

d) The epicuticle (arrow) of the general epidermis during an early formative stage. The epicuticle is seen to be an homologous structure with the lining of the trichogen duct seen in the previous plate. (cf Locke, 1966)

a)

b)

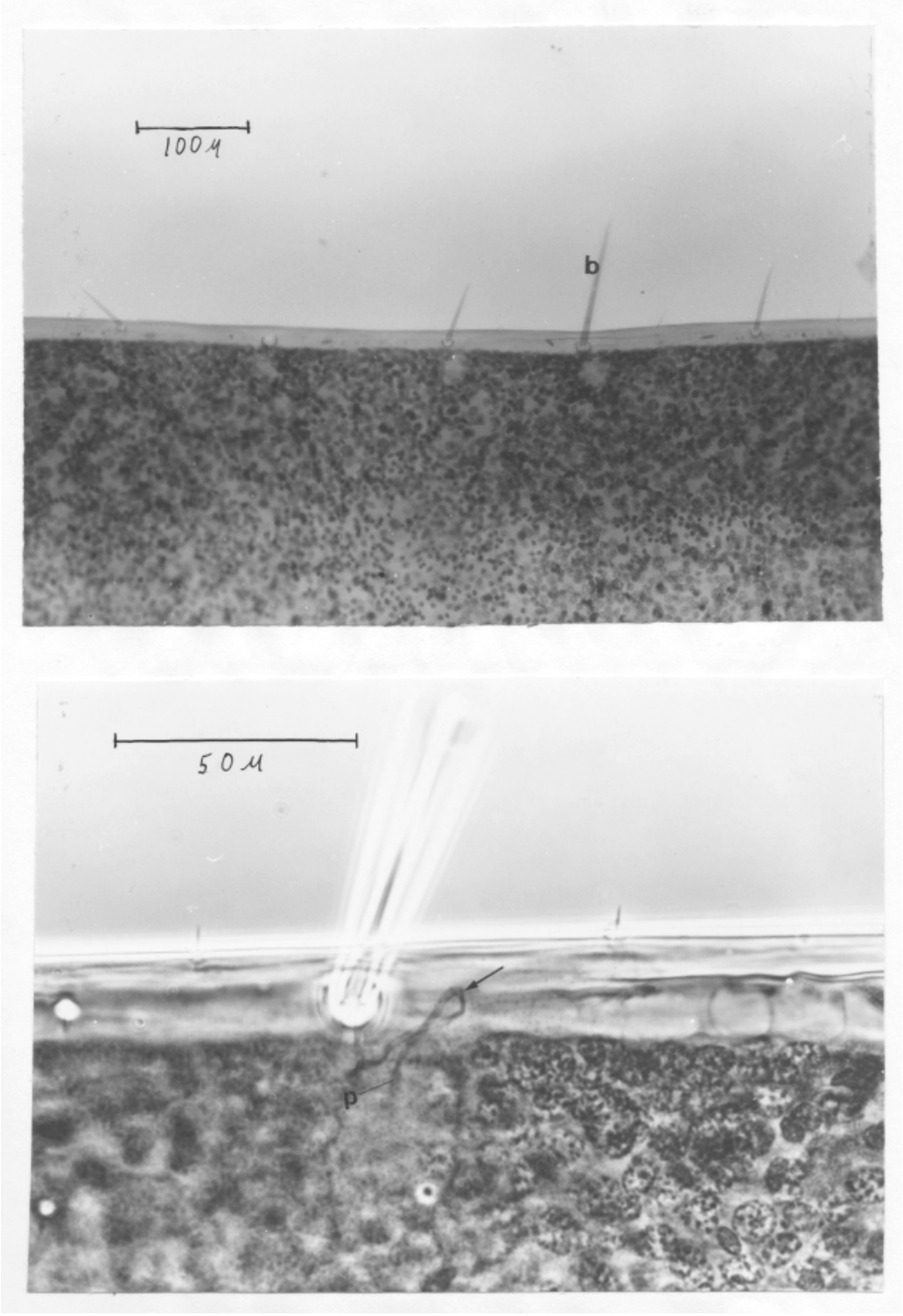


Fig. 9 a-f Bristle Morphogenesis (wholemounts, g (longitudinal section)

a) The hind edge of a tergite showing the bristles b and associated formative nucleus within the epidermis.

b) A nerve coil (arrow) is seen extending from the epidermis to the new bristle. The first stage of producing a new bristle is seen as a, protuberance (p) at the proximal end of the nerve.

c)

d)

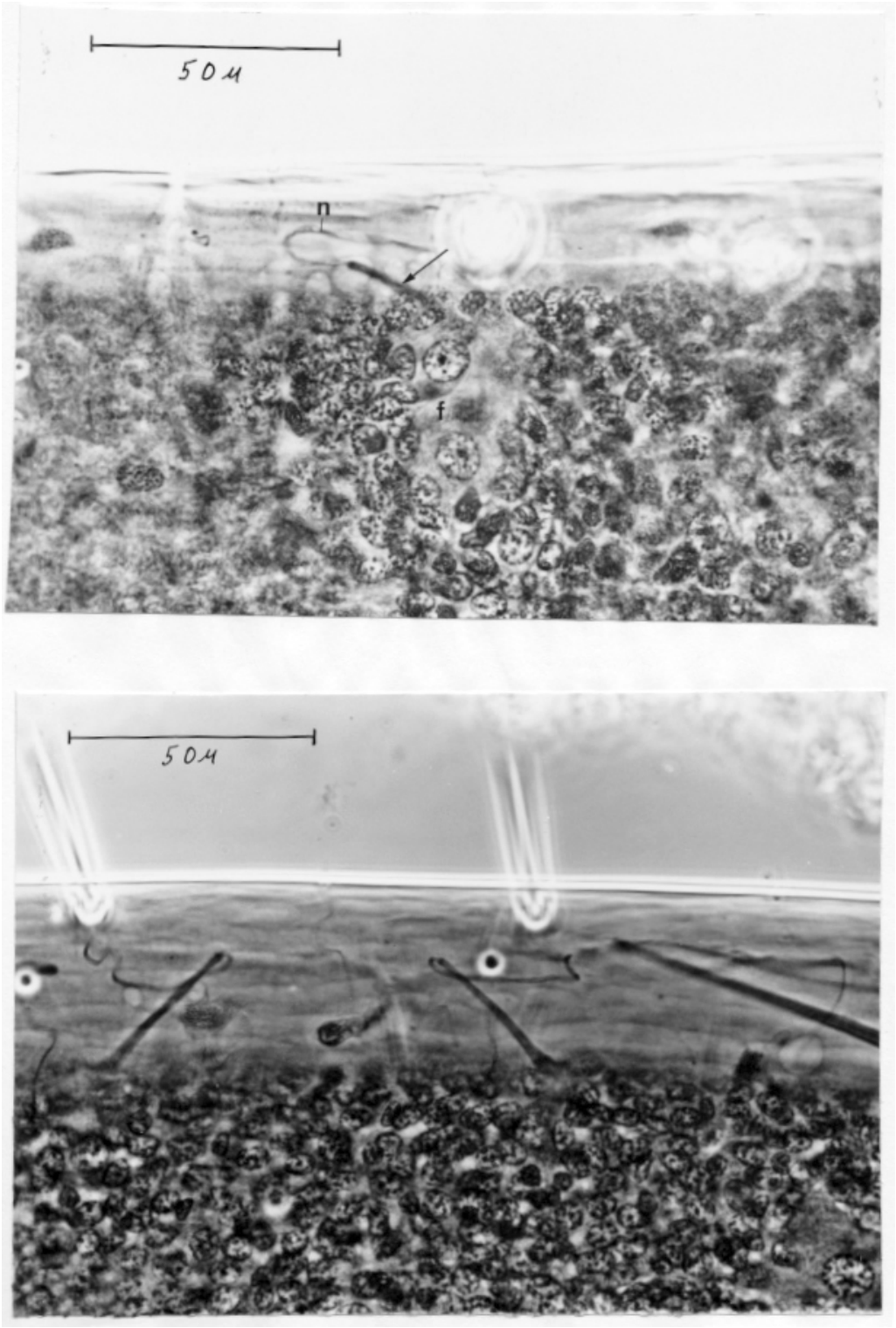


Fig 9 cntd.,

c) The new bristle (arrow) is seen to have grown and the cytoplasm of the formative cells (f) are dearly seen. The nerve coil (n) extends back to the socket of the old bristle (b) which is broken off in this preparation.

d) The new bristle must grow laterally in the molting space since its length is usually greater than the depth of the molting space.

e)

f)

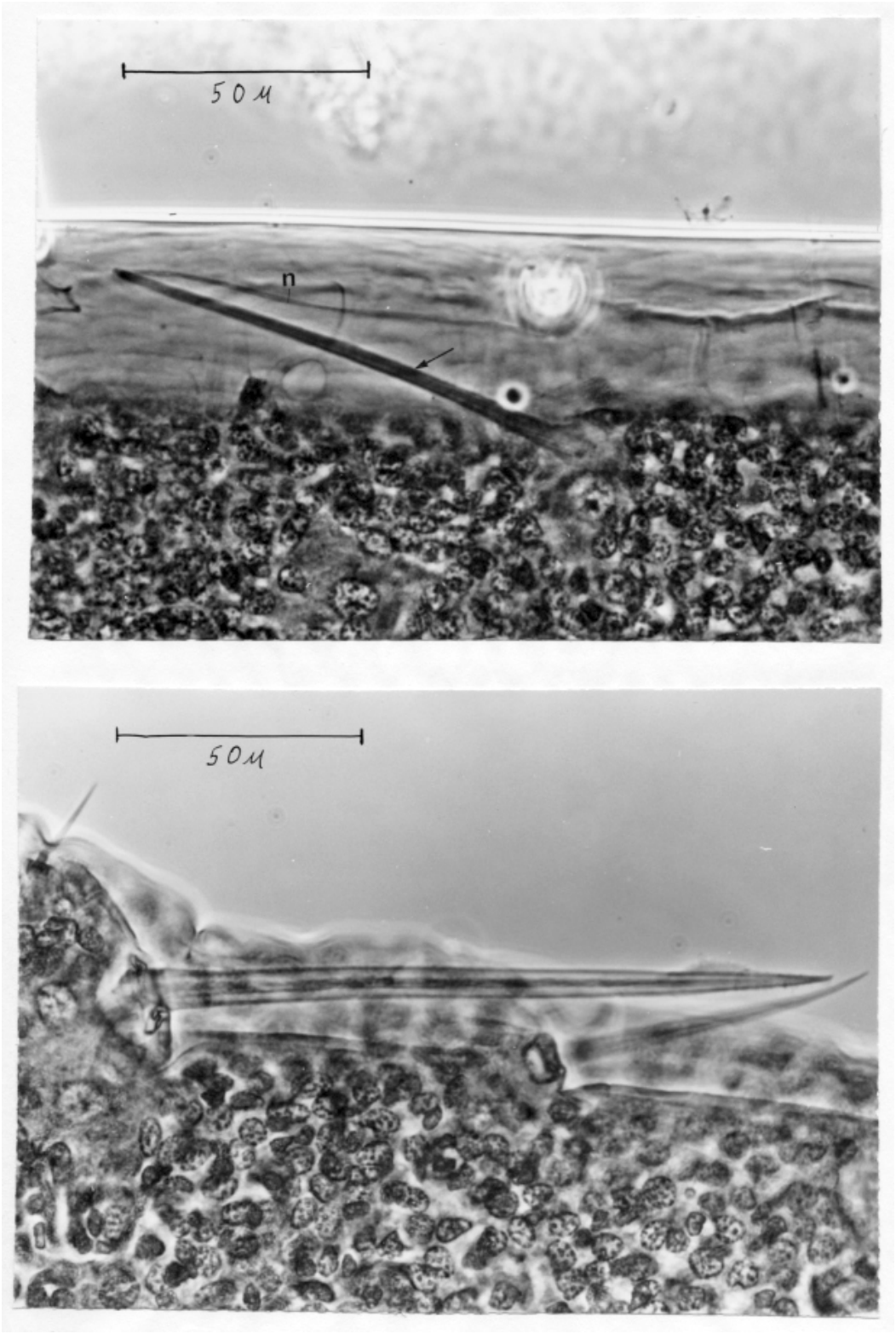


Fig 9 cntd.,

e) The growth of the new bristle (arrow) laterally necessitates a length of nerve coil (n) sufficient to reach back to the old bristle if nervous connection with the environment is to be maintained.

f) A completed bristle is shown with the old cuticle removed. The nerve coil was removed with the old cuticle.

g)

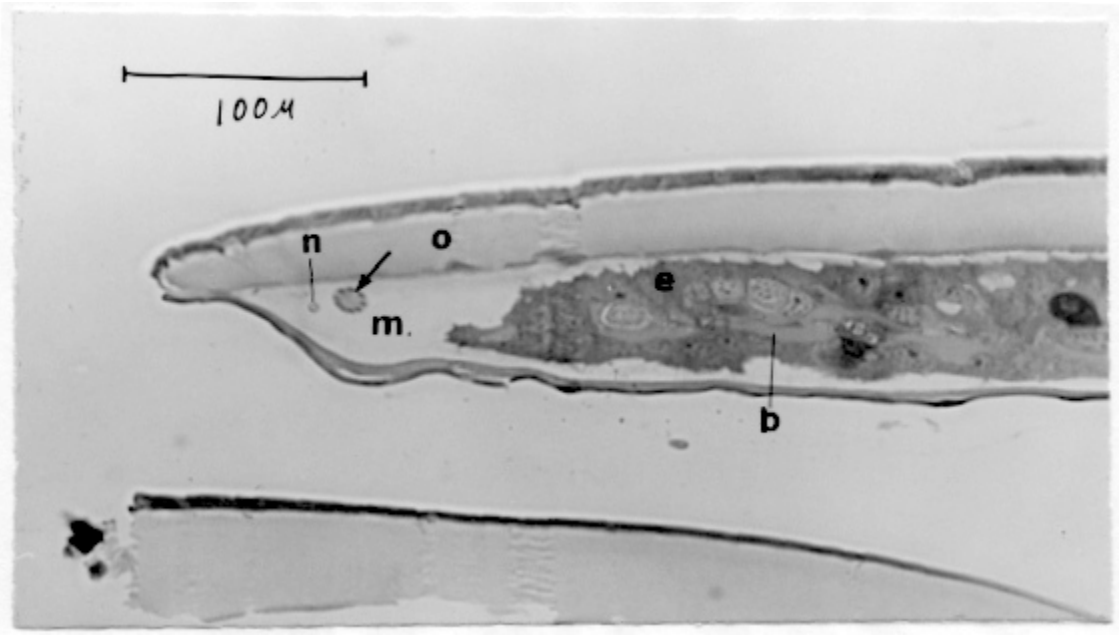


Fig 9 cntd.,

g) A longitudinal section through the hind edge of a tergite showing the old cuticle (o), the epidermis (e), the molting space (rn), blood space (b), a cross section of a new bristle (arrow) and its nerve coil (n) which in serial sections extends back to the old bristle.

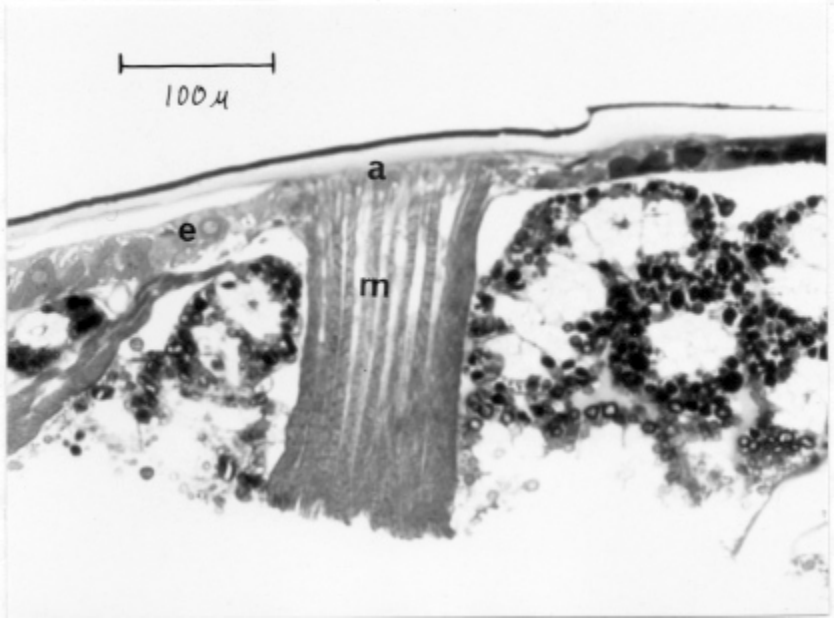


Fig. 10. The muscle attachment (a) of a dorsal-ventral abdominal muscle (rn) to the fourth abdominal segment tergite in a fourth instar *Blattella germanica* nymph. The other end of the muscle would be attached to the fourth abdominal sternite. The epidermis (e) at this point in time is seen to be detached from the old cuticle except at the muscle attachment.

The rationale for choosing the above events was to find a range of morphological and physiological events spread throughout the stadium and to interpolate some of the well-known hormonal events into this sequence as a basis for experiments on causal relationships. Of particular

interest was the relationship of the regeneration critical period (Def. 3) to the hormonal events associated with the head and prothorax critical periods (Def. 1 and 2). The effect of regeneration on other morphological events of the molting cycle (Def. 5-10) was also of interest, since regeneration is known to delay the ecdysis.

The first problem, then, was to establish a map of the events of the molting cycle. This was done in a number of experiments. Each experiment concentrated on a limited number of the events. The experiments are summarized in Table 8 and in temporal map form in Figure 11. Some comments on Table 8 are necessary. Since all the experiments were performed at different times, the T_{50}'s for a given event vary from experiment to experiment due both to incubator effects and between-experiment effects and therefore they cannot be directly compared. However each experiment has a sufficient overlap of events to allow a reasonable inference about a composite map of the events studied, which is given in Figure 11. Also it must be noted that parameters of the event ecdysis were not measured by probit analysis but by the conventional formulae of estimating the mean and variance for quantitative variables; for this reason the test of parallelism of probit could not be performed between data from ecdysis and data from other events whose parameters were estimated by probit analysis.

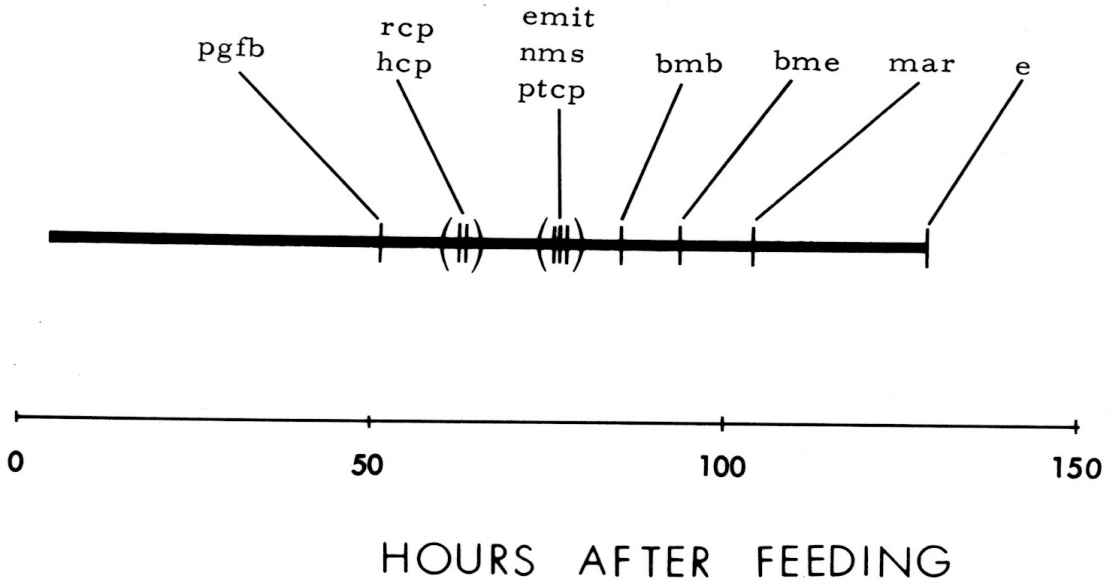


Fig.11. Map of molting cycle events determined by probit analysis.

1. *pgfb.* = protein granules in fat body
2, 3. | *rcp.* = regeneration critical period
| *hcp.* = head critical period
4, 6. | *emit.* = end of epidermal mitosis
| *nms.* = nerve coils in molting space
| *ptcp.* = prothorax critical period
7. *bmb.* = bristle morphogenesis beginning
8. *bme.* = bristle morphogenesis end.
9. *mar.* = muscle attachment release
10. e. = ecdysis

Table 8. Results of Probit Analysis.											
Exp.		**Event**				**Estimated Parameters**			**Tests of Significance p[4]**		
No.	**Strain**	**No.**	**Name**	**N**	**N_W[1]**	**T_{50}**	**Sx[2]**	**S_{t50}[3]**	**Normal**	**Parallel**	**Sequence**
1	NY	4	*p.g.fb*	20	5	57.9	7.6	3.5	>0.5	<0.01	-
		3	*c.p.reg*	400	218	72.4	12.0	0.6	>0.1		
2	NY	9	m. att.	650	249	174	7.4	0.52	>0.1	-	-
		10	ecdysis	650	-	200	8.0	0.3	>0.5		
3	NY	9	m. att.	360	196	172	5.5	0.6	>0.5	-	-
		10	ecdysis	40	-	200	7.0	0.35	>0.05		
4	ORL	3	*c.p.reg*	152	50	57.8	10.6	2.8	>0.5	>0.1	<0.01
		4	*p.g.fb*	100	43	75.0	10.8	1.7	>0.5	-	-
		10	ecdysis	47	-	125.2	12.3	1.7	>0.05		
5	CR	1	head.c.p.	87	42	76.6	10.8	1.75	>0.20	>0.5	>0.8
		3	*c.p.reg*	274	114	77.0	8.7	0.9	>0.5	>0.5	<0.01
		2	*pth.c.p.*	91	24	87.4	8.6	3.0	>0.5		
		10	ecdysis	35	-	136.1	11.8	1.9	>0.05		
6	ORL	3	*c.p.reg*	264	131	62.8	9.8	0.86	>0.5	>0.5	<0.01
		2	*pth.c.p.*	270	128	68.4	9.9	0.87	>0.2	>0.5	<0.05
		6	nerve c.	122	47	66.4	9.6	1.9	>0.5	-	
		7	bris dev+	122	51	73.7	7.4	1.0	>0.5	-	
		8	bris dev-	122	45	86.3	10.2	1.7	>0.5	-	
		10	ecdysis	169	-	121.8	8.5	0.7	>0.05		
7	ORL	3	*c.p.reg*	173	137	68.5	9.3	0.93	>0.5	<0.01	-
		2	*pth.c.p.*	127	60	94.4	24.5	7.8	>0.5	-	-
		10	ecdysis	120	-	124.3	10.2	0.93	>0.01		
8	*Periplaneta*	3	*c.p.reg*	264	108	83.7	14.8	1.4	>0.05	-	-
		10	ecdysis	54	-	171	17.0	2.3	>0.05		

[1] Nw = reduction of sample number (N) due to using probit analysis
[2] Sx = standard deviation of the event in time.'
[3] Standard error of the estimate of the T_{50} of the event.
[4] p = Probability of no significant difference.

An example of an experiment which was thrown out is seen in experiment 7 of Table 8. In the first place, the test of parallelism rejects that the *c.p.reg* and *pth.c.p*. are parallel events; therefore no test of sequence could be made. This was probably due to a high mortality rate in the *pth.c.p*. assay. In other experiments (5, 6), using an improved method of ligation, the mortality was negligible, the events were parallel and test of sequence could be performed. In the same experiment 7 of Table 8 the normality of

the data on ecdysis was rejected at the 0.01 level of significance; this throws some doubt on the validity of that probit analysis for mapping events in this particular culture of animals. In experiment 6 of Table 8 the assays of the events 6 to 8 were done on the same set of animals and therefore the three events were not measured by independent samples; as a result a test of parallelism and sequence could not be performed on them since probit analysis demands independent samples for the parallelism test. However the other events in the same experiment were assayed by independent samples to which the tests of parallelism and sequence could be applied.

The map of the molting cycle in Figure 11 shows the events whose sequence has been determined as well as those events whose sequence could not be resolved using probit analysis. This does not mean however that the unresolved events are simultaneous. The resolution of probit analysis in mapping events depends upon the synchrony of the cultures of animals and the number of animals used in the assay of an event. With a number of assumptions to simplify the formula, the number of animals (n) needed to resolve two events d hours apart at the 5% significance level using a colony of animals with a variance, s, in physiological age is given by,

$$n = 16\, s^2/d^2$$

The *Blattella* strain ORL has a standard deviation of ecdysis, s, of about 8 hours. Computing the number of animals needed to resolve events 1, 1/2, 1/4, or 1/10 hour apart we get 1,024; 4,096; 16,384; 102,400 animals needed respectively to get the desired resolution.

It would seem impractical therefore to try to resolve events which are much closer than an hour apart. This method however has given a first approximation to a map of developmental events in the individual.

The map of Figure 11 shows that the T_{50} of the critical period for regeneration is not resolvable from the T_{50} of the critical period for the head. (Actually the difference in the T_{50}'s is given by the 95% C.I. = 0.4+/- 3.4hrs.) This is already suggestive that regeneration feeds back on the control of molting via the brain. The map also shows that mitoses in the epidermis occur before the critical period for the prothorax. This is suggestive that the prothoracic gland is needed up to the time when mitoses stop in the epidermis in order to insure a molt.

The end of mitoses in the epidermis occurs quite close to the first appearance of nerves in the molting space, the sequence of these two events was not tested by probit analysis because simultaneous observation of the two events could be observed in a whole mount of an individual animal and it offered a better chance to study the correlation of the two events within individuals as opposed to independent samples. These two events are also of interest because the T_{50} of the appearance of nerve coils in the molting space could not be resolved from the T_{50} of the prothorax critical period.

Disappearance of mitoses and appearance of nerve coils then, are possibly among the first events of the molting cycle to be independent of further presence of the prothoracic glands which are the source of the molting hormone. For this reason they deserve further attention.

2. The Correlation Between the End of Epidermal Proliferation and the Appearance of Nerve Coils

A synchronous culture of fourth instar ORL strain was sampled at 6 times which were known to span the times when mitoses in the epidermis were stopping and nerve coils were appearing in the molting space. At each time a sample of 20 animals was taken and the abdominal tergites were fixed in Bouins, subsequently stained specifically for DNA by the Feulgen nucleal reaction, and prepared as

whole mounts. Each whole mount of an animal was assayed for both events, (end of mitoses = (-) if mitoses are still present or (+) if mitoses have stopped; appearance of nerve coils = (-) if they have not appeared or (+) if they are present). Thus a given animal can be represented by a vector of the two events V = end of mitoses, appearance of nerve coils. This vector is a function of time, t, and V(t) in an individual will start at (- , -) when neither of the events have occurred within the individual. If the end of mitosis occurs before the appearance of nerve coils in an appreciable number of cases then the intermediate V(t) = (+, -) will appear and a similar argument holds for the other alternative V(t) = (-,+). If both events were simultaneous then no intermediates between (-, -) and (+, +) would be found.

The numbers of each type of animal observed at time 't' spanning the major part of the range of the events is given in Table 9.

Table 9

V(t) =(mitoses;nerves)	Sampling times(t) I	II	III	IV	V	VI
(-, -)	20	18	6	6	0	0
(-, +)	0	1	1	0	1	0
(+, -)	0	0	2	3	1	0
{+, +)	0	1	11	11	18	20

This table shows that the two events are correlated in time since the intermediate combinations of events occur with such a low frequency. The occurrence of intermediates of both types has to be accepted with a certain amount of caution. The assay for the nerves is quite clear and reliable but the assay for the end of proliferation carries the possibility that a false positive assay may occur. Since at the end of proliferation of the epidermis mitoses may occur at a very low frequency it is possible that mitoses could be absent from an animal at the time of fixation although mitoses were still to come at a very low frequency.

However this only throws doubt on the intermediate animals of type (+,-). The animals of type (-, +) can not be disputed; they definitely have not stopped mitosis in the entire epidermis and yet they do show the earliest signs of nerve coils in the molting space. It must be emphasized though that (-, +) and (+, -) animals were always in the very last stages of epidermal proliferation. This experiment demonstrates that when considering the entire tergite and intertergite region, nerve coils appear in the molting space at the hind edge of the tergite before mitoses have completely stopped.

3. Epidermal mitosis during the molting cycle

The first experiment on cell proliferation was aimed at estimating the increase in cell number in the epidermis during the molting cycle. A synchronous culture of fourth instar nymphs (sd=12 hours at ecdysis) was sampled at intervals starting at 12 hours after feeding.

The abdominal tergites were fixed in Bouins, stained by the Feulgen technique and prepared as whole mounts. The intertergite was chosen as a region for counting cells because it has no dermal glands or bristles (Fig. 12). These structures have more than one nucleus and make counting difficult.

a)

b)

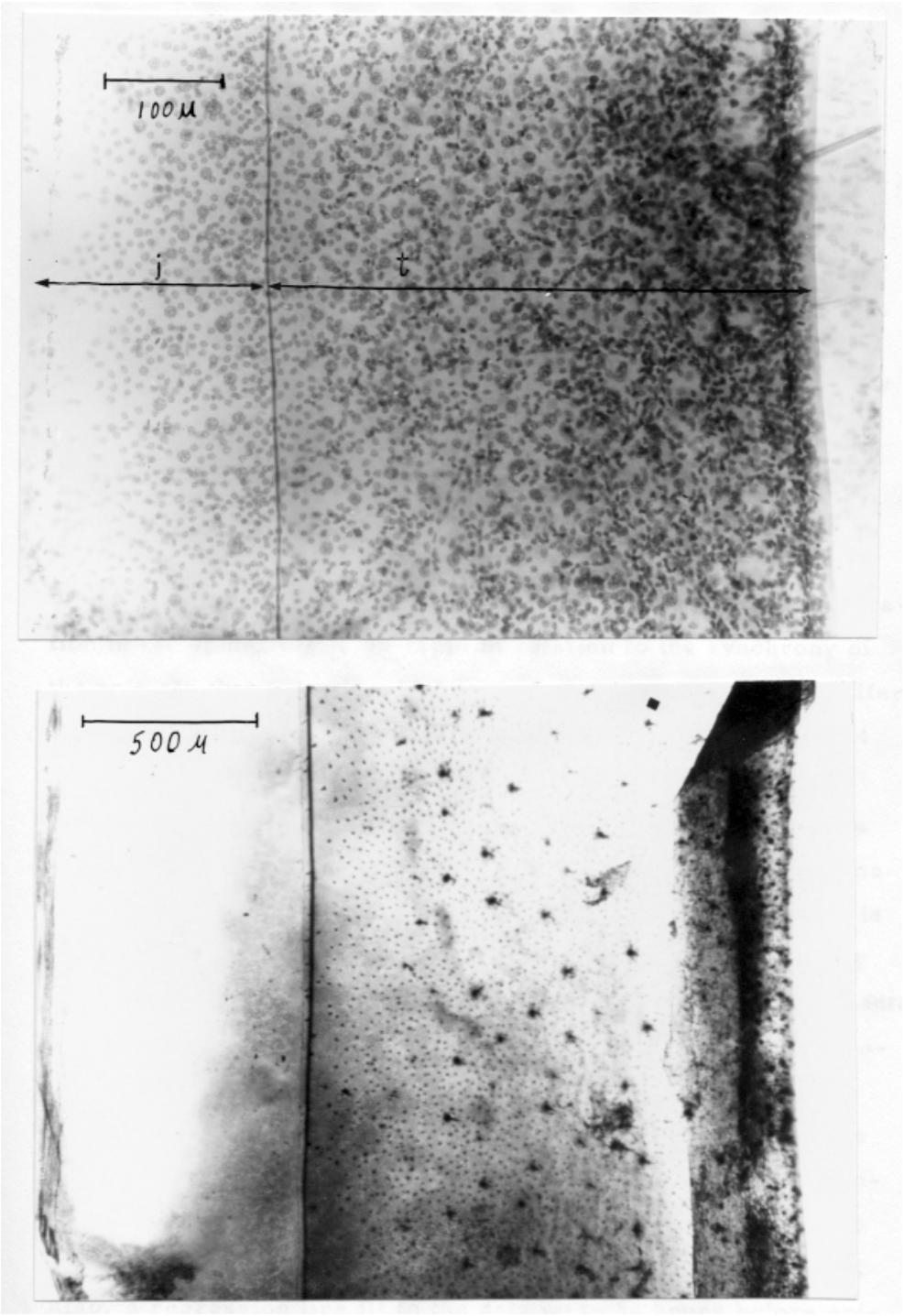


Fig. 12. Abdominal tergites showing the absence of dermal glands from the intertergite region.

a) A Feulgen-stained whole mount of an abdominal tergite (t) and intertergite (i) region of *Blattella germanica* showing the relatively evenly dispersed nuclei of the intertergite region as opposed to the more confused tergite epidermis.

b) An ammoniacal-silver nitrate preparation of an abdominal tergite and intertergite region of *Periplaneta americana* parallel to the above illustration showing the canals of dermal glands as evenly spaced small black silver deposits and bristles as larger silver deposits. The intertergite region is seen to lack dermal glands and bristles.

A standard area, of the intertergite was chosen, a *camera lucida* trace of the nuclei within this area was made for each animal, and the number of nuclei were counted and plotted as nuclei per unit area against the time of assay (Fig. 13). The figure shows the practical problem of studying quantitative variables in a "synchronous" system. When a quantity is changing slowly in time the variation can be treated by linear regression techniques assuming that the quantity is distributed normally about some mean at each given time. When a change is occurring relatively rapidly in relation to the degree of synchrony however, the observations are not normally distributed about the mean at a given time. This is seen in Figure 13 in which at 63 and 70 hours the observations are grouped about two different means for each time. Another way of looking at it is that cell proliferation in the epidermis is so rapid in relation to the synchrony of the animals that animals which have either not begun cell proliferation or ones which are finished are seen most often. Figure 14 shows the particular region studied in Figure 13 with a large number of mitoses; these were rare animals (2 of 40). This is an important point because in the experimental sections to come it must be borne in mind that when an experimental treatment is applied to a group of synchronized animals, these animals may vary considerably in their state of progress of rapid developmental processes. A practical aspect of this graph is that the cell proliferation cannot be studied meaningfully by linear regression, since the observations are far from normally distributed at the times when cell proliferation is occurring. The data do demonstrate that at the end point of proliferation in the epidermis the cell density is approximately doubled over the starting density. Also, a regression line fit to the data up to 54 hours before epidermal mitosis begins shows a decrease in cell density with time which, although not a statistically significant decrease, is substantiated by some subjective observations on the whole mounts.

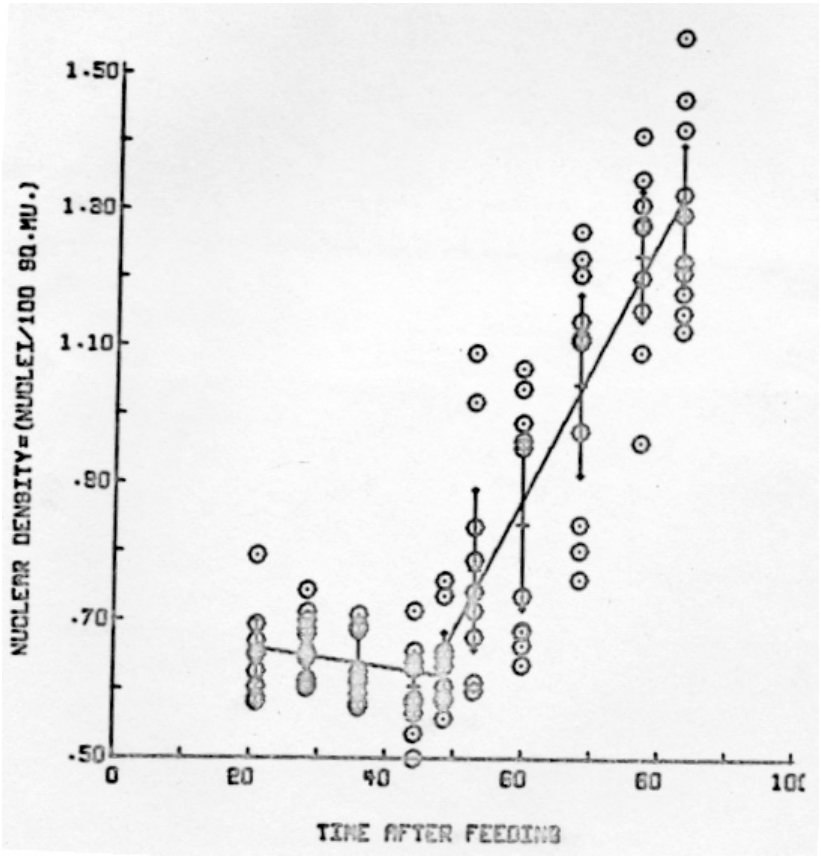


Fig. 13. Nuclear density of the intertergite region of *Blattella germanica* as a function of time after feeding. Individual observations are plotted with an indication of the mean for each time and a 95% C.I. for the mean.

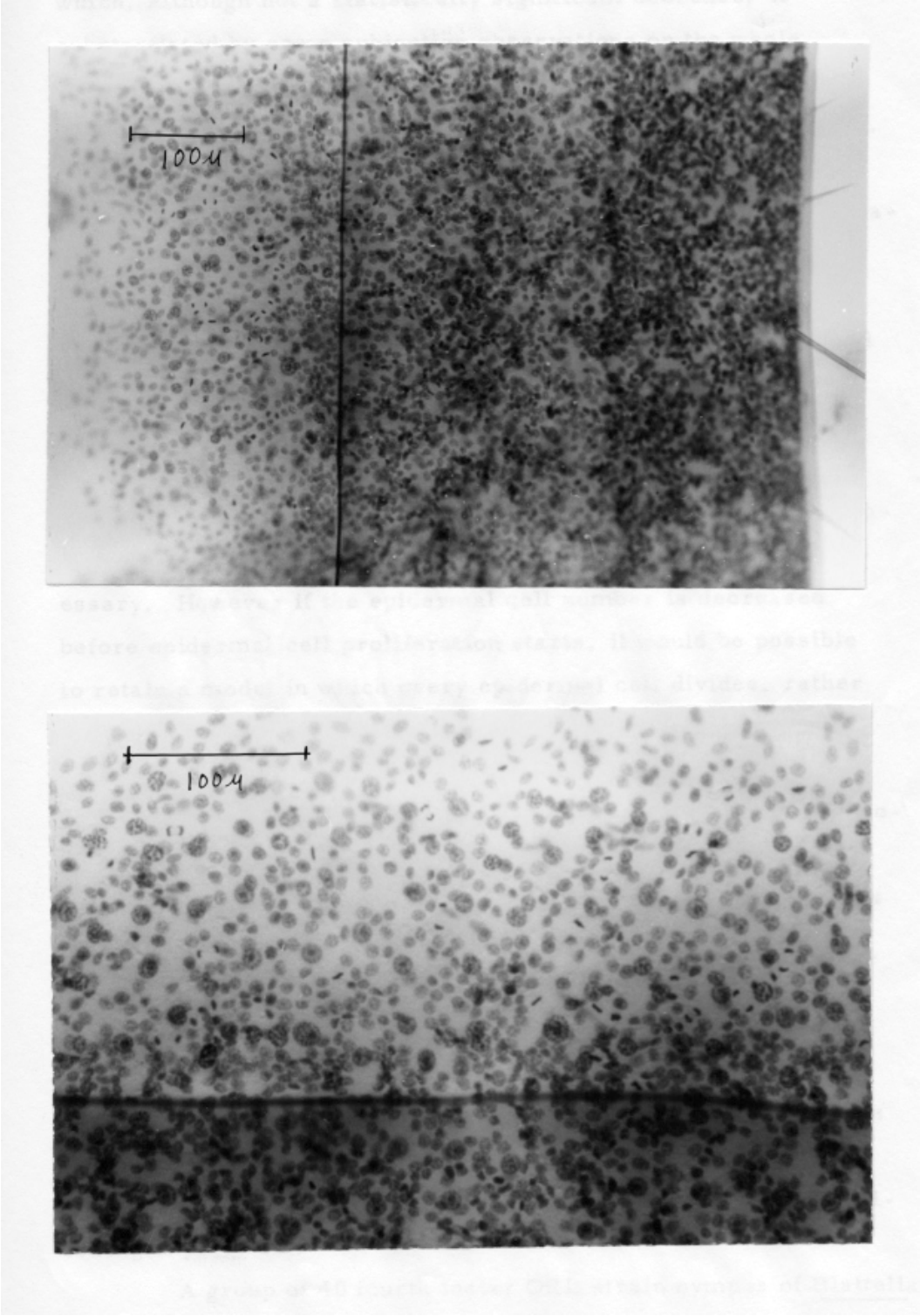


Fig. 14. Two animals exhibiting a high density of mitoses in the intertergite region. Feulgen stained whole mounts.

a) Whole tergite/intertergite showing lack of organules in intertergite region.

b) Intertergite region above margin to show small epidermal nuclei and large oenocyte nuclei.

Dying epidermal cells with clumped nuclei are seen in the epidermis at the time the decrease would be going on (Fig. 15a). Also colchicine collection of mitoses over periods of 2, 4 and 8 hours during the period when the epidermis is not proliferating, do pick up scattered groups of mitoses which are probably differentiations of sensory organs (Fig. 15b,c) in the cuticle. Both of these factors could result in a decrease in the epidermal cell density before the main cycle of epidermal mitosis begins.

This decrease in cell numbers makes sense for the following reasons. While the animal about doubles in size with each molt, this is only results in an increase of $2^{(2/3)} = \sim 1.59$ in area. Doubling of epidermal cell number at each molt is therefore not necessary. However if the epidermal cell number is decreased before epidermal cell proliferation starts, it would be possible to retain a model in which every epidermal cell divides, rather than a model in which some cells divide and others do not.

To pursue further the problem of whether every cell divides, colchicine was used in an attempt to collect as many proliferative mitoses as possible as colchicine mitoses. It was found that 5×10^{-5} M colchicine would continue collecting mitoses for up to and beyond eight hours, but after eight hours some of the colchicine mitoses, presumably the first to form, disintegrate. At lower doses, 2×10^{-5} M and lower, bipolar mitoses start reappearing by eight hours. Therefore eight hours of colchicine treatment is the maximal time length over which mitoses can be collected with assurance that one would see all the cells which were collected. This information helped me set up the following experiment.

A group of 40 fourth instar ORL strain nymphs of *Blattella germanica* which were predicted by the developmental map to be in the active stage of epidermal proliferation were injected, with 5×10^{-5} M colchicine. It was hoped that some of the animals would be in an early stage of epidermal proliferation so that every subsequent epidermal mitosis would be collected as a colchicine mitosis over the next eight hours.

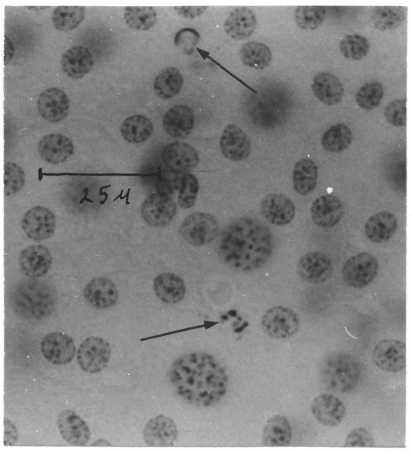
After eight hours the animals were sacrificed and Feulgen-stained whole mounts of their abdominal tergites were prepared. Varying numbers of epidermal cells appeared as colchicine mitoses. Figure 16 a,b shows the tergite of one animal which had almost every epidermal cell in division in some regions. The limit of eight hours of collection using colchicine prevents making a definite conclusion on whether every epidermal cell divides.

4. The Pattern of Epidermal Mitoses in Time

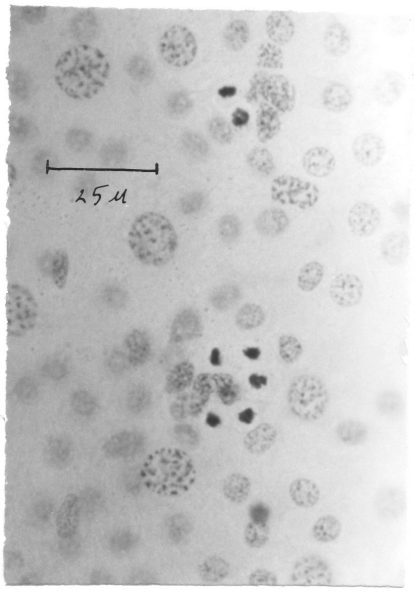
An attempt was made to demonstrate the pattern of mitosis in the epidermis and its relationship to the first visible signs of molting. Two types of colchicine experiment were performed, one varying the time of collection and another the duration of collection of mitoses. The first used a fixed duration of colchicine mitosis collection applied at different times after feeding. Samples of 10 animals were taken at various times from 0 to 100 hours after feeding, injected with 5 x 10^-5 M colchicine, incubated for 4 hours and then the abdominal tergites were fixed, stained with Feulgen and prepared as whole mounts. The pattern of density of colchicine mitoses in these animals is given in Figure 17. Four types of situation are included in the figures:

1) the early type of grouped mitoses which are most likely differentiating organelles such as dermal glands and sense organs (Fig. 15b).
2) a high density of colchicine mitoses (Fig. 16b).
3) a low to medium density of' colchicine mitoses
4) no or practically no colchicine mitoses (Fig. 12).

a)



c)



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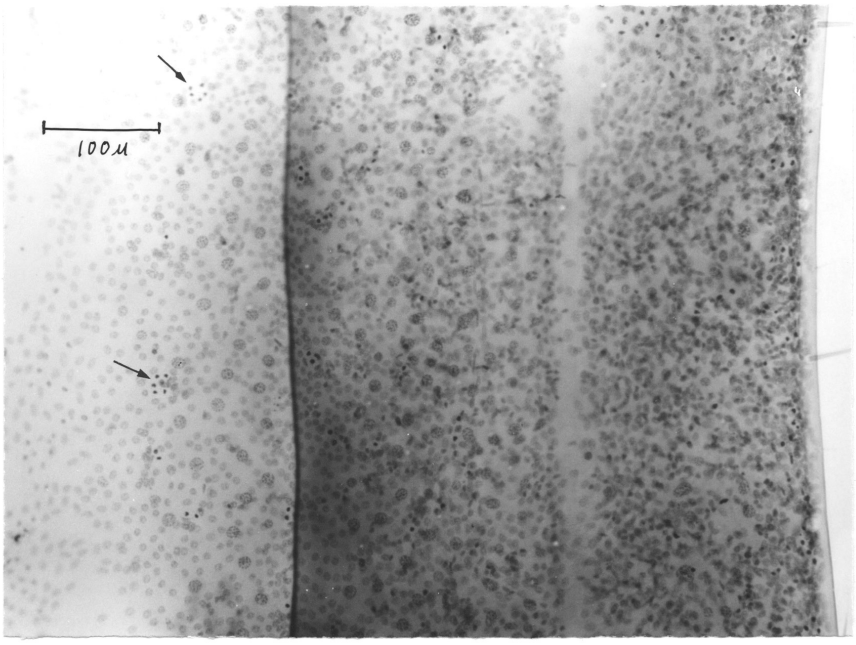


Fig. 15. Contributing factors to the reduction of epidermal cell density in the intertergite region.

a) (left) Dying epidermal cells in the intertergite region appear as Feulgen positive fragments and globules (arrows).

b) (below) A whole mount of the tergite-intertergite region of an abdominal segment of *Blattella germanica* eight hours after injected to 5 x 10^{-5} colchicine. The injection caught the animal at a time when epidermal proliferation had not started yet and only differentiative mitoses occur as seen by the clusters (arrows) of colchicine mitoses mainly in the intertergite region.

c) (right) Higher magnification of a region of Fig. 15b showing the cluster of colchicine mitoses about another group of cells which presumably divided previous to the injection.

These categories are subjective but they make understanding the pattern of mitoses easier. They are however an over-simplification since there are intermediate grades between categories 2, 3, and 4.

Within an animal each abdominal tergite shows the same pattern of colchicine mitoses as neighboring tergites, which is additional evidence that the patterns are real. An observation which can be made immediately is that general mitosis of the epidermis starts at the posterior of each segment and ends at the most anterior part of the segment. Figure 18 gives some demonstrative whole mounts to this point.

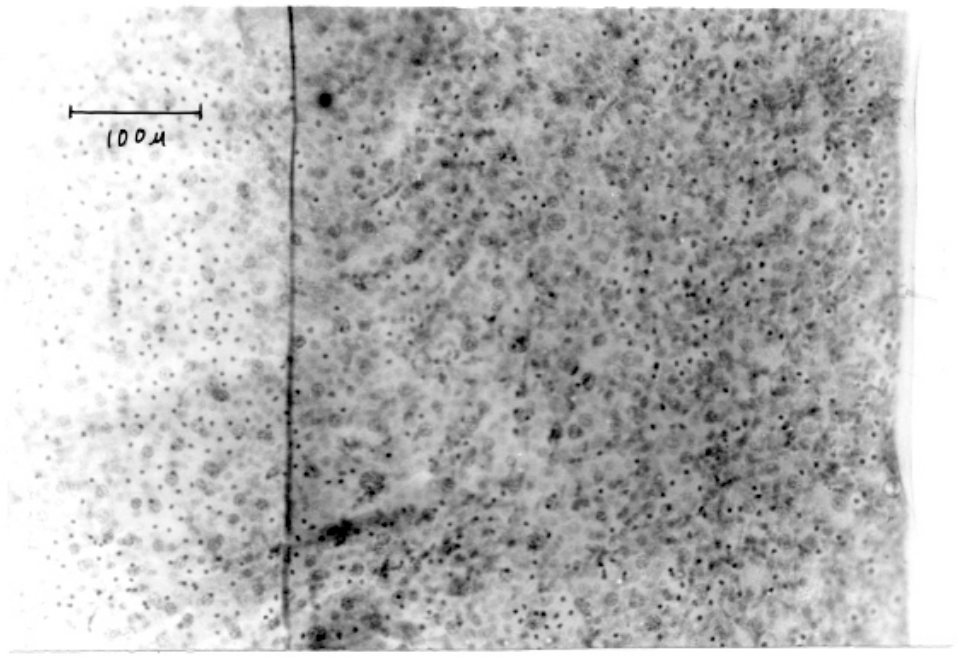
Another observation from Figure 17 is that another starting point of proliferation exists at the border between the tergite and intertergite. This starting point starts to show colchicine mitoses only after mitoses are well begun at the posterior edge of the tergite. We may conclude from this experiment that mitosis starts at the rear of a tergite and proceeds anteriorly, although it does not do so linearly. There seems to be some interruption or new starting point at the tergite-intertergite border.

A second type of colchicine experiment was used to determine the relation of differentiative type mitoses to proliferative divisions. Colchicine was administered at a fixed time when the majority of animals were predicted to be starting the stage of active epidermal proliferation. Twenty animals were sacrificed at 1, 4 and 8 hours after colchicine was administered and prepared as Feulgen-stained whole mounts. With the 1 hour collection I could tell what regions of the epidermis were dividing within one hour of each other and similarly with the eight hour collection I could tell which regions were dividing within 8 hours of each other. Combining the two collection period lengths I could tell within certain time limits the time interval between the start of one region and another. The time when the organule type divisions in the intertergite region (Fig. 15b,c) stopped in relation to the beginning of proliferative divisions was of particular interest in this experiment.

Only in the 8 hour collections were both organule type mitoses and epidermal mitoses at the tergite-intertergite border. This indicates that organule differentiation stops at most 8 hours and probably more than 4 hours before proliferative division starts. In a similar experiment aimed at determining the relation between the end of mitoses and the appearance of nerve coils I injected colchicine at a time when the animals were predicted to be finishing epidermal mitoses. Again 20 animals were fixed at 1, 4 and 8 hours after injection. Both the 4 and 8 hour groups showed colchicine mitoses at the rear edge of the tergite at the same time as nerve coils were observed in the molting space, but the one hour treatment group did not show colchicine mitoses in the same region as nerve coils were found. When nerve coils were present in the trailing edge of the tergite the only part of the animal which had colchicine mitoses was the intertergite region. This resolves the problem posed in Table 9. It seems that mitoses can still occur in the intertergite region while at the trailing edge of the tergite the epidermis is detaching from the old cuticle and nerve coils are appearing in the molting space. But mitosis stops at least locally before the nerve coils appear in the molting space.

We may conclude from this section on epidermal mitosis and the start of molting that the earliest signs of molting activity, i. e. the appearance of nerve coils in the molting space, occur only after mitoses have ceased to appear nearby. Mitoses associated with the intersegmental muscle attachments to cuticle do persist in appearing in the anterior of the segment after the nerve coils appear at the posterior. Since the muscle attachments are the last parts of the epidermis to detach from the old cuticle, about 25 hours before ecdysis, they are perhaps also slower in completing their mitoses. However the late mitoses at the anterior edge of the intertergite are more probably associated with their anterior position in the segment rather than their being muscle attachments, since epidermal cells associated with dorsal-ventral muscle attachments in the lateral part of the tergite are not late in their mitoses.

a)



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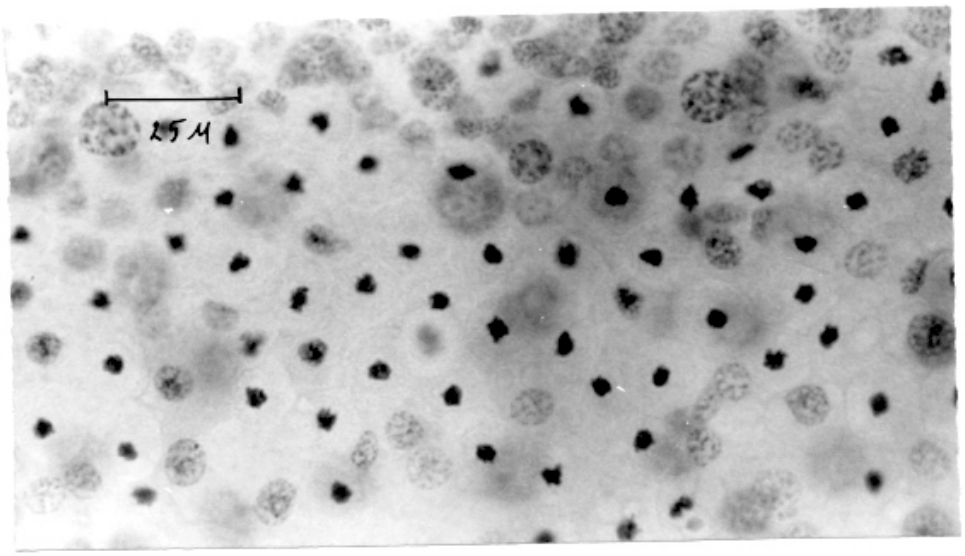


Fig. 16. The abdominal tergite of an animal injected with 5 x 10^{-5} M colchicine and sacrificed after eight hours. The intertergite shows a high density of colchicine mitoses.

a) The entire tergite intertergite region.

b) Higher power of the intertergite region showing almost total division of the epidermal cells.

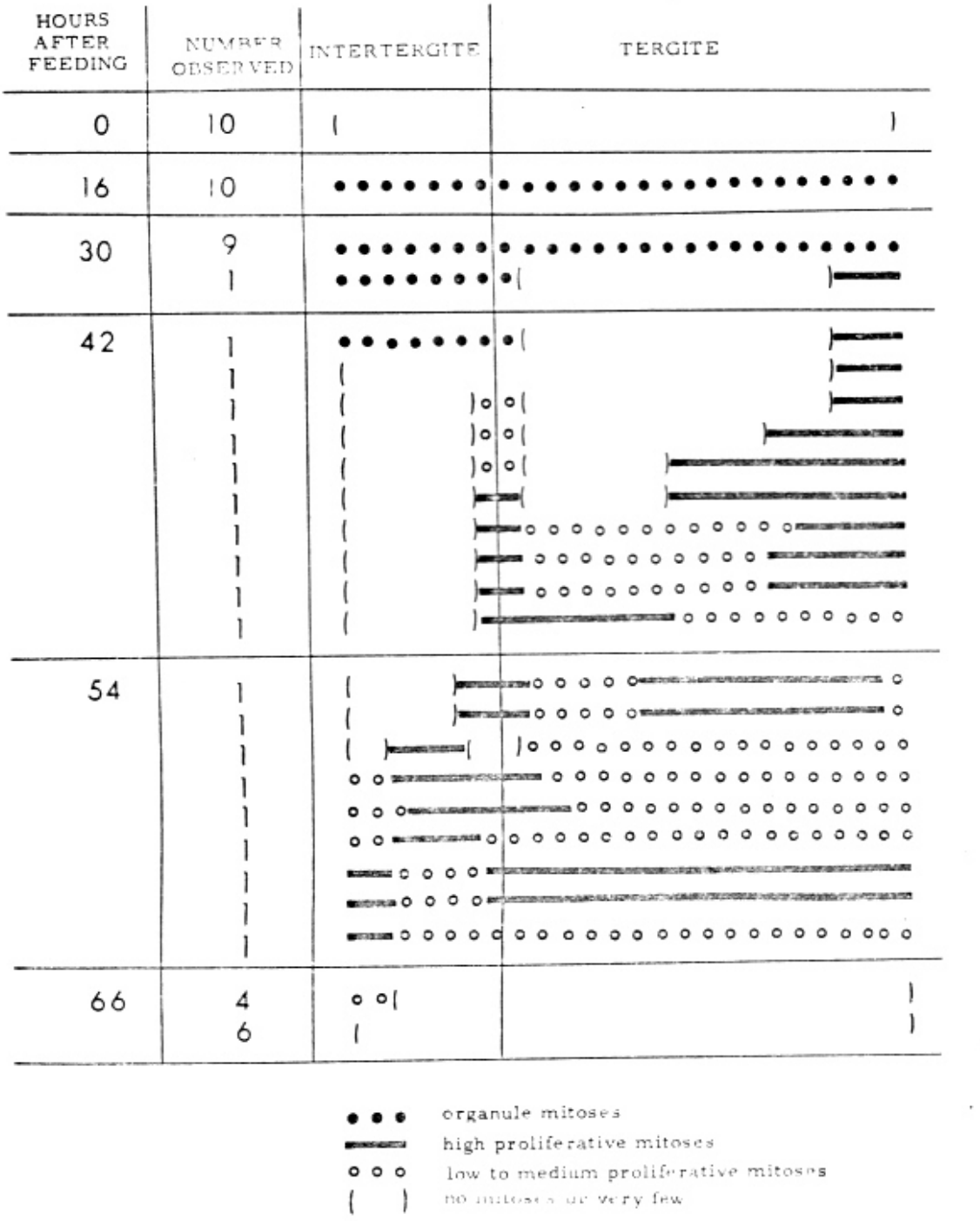


Fig. 17. Patterns of colchicine mitoses collected for 4 hours at various times after feeding. A sample of 10 animals was used at each time after feeding.

a)

b)

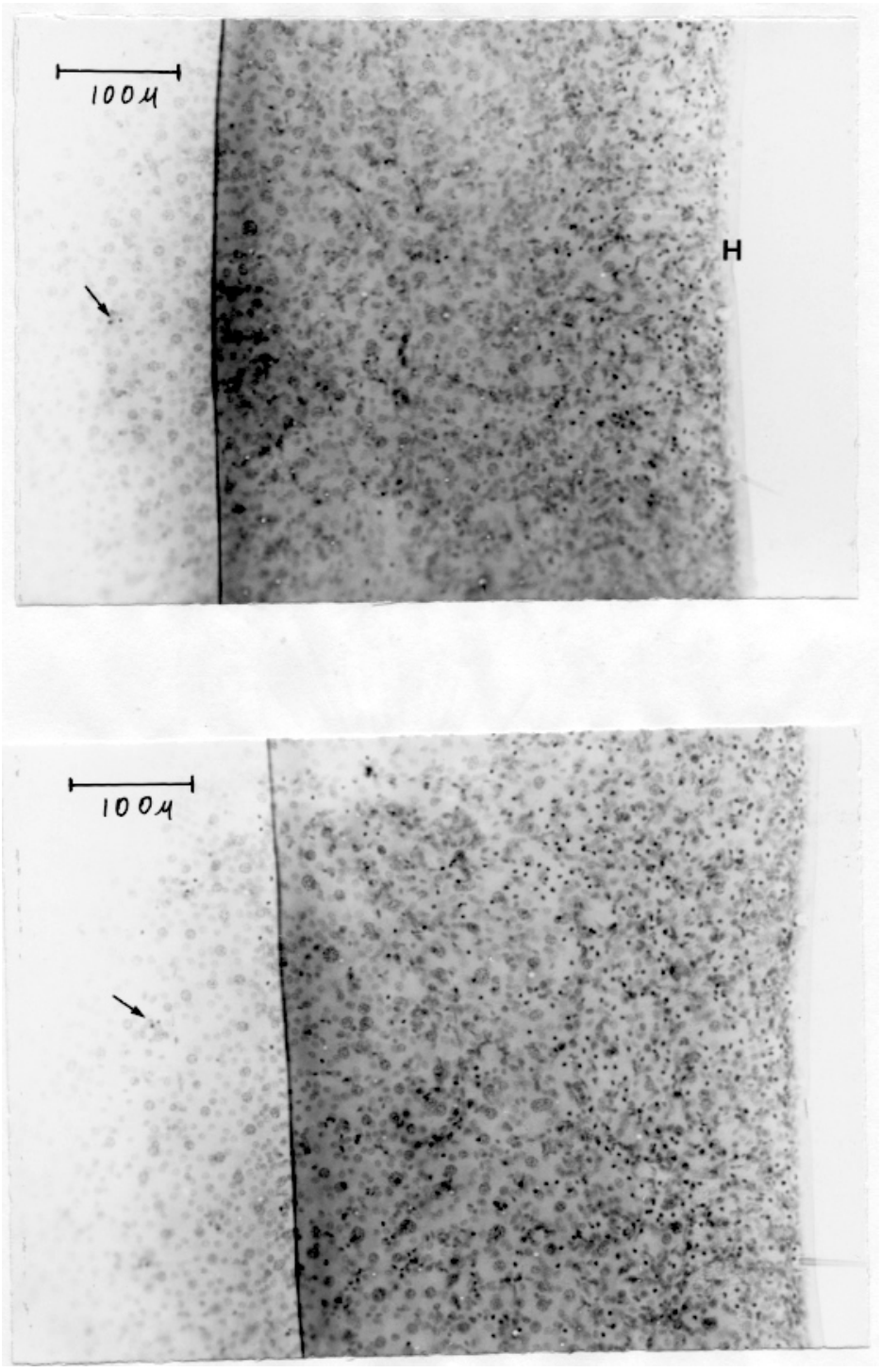
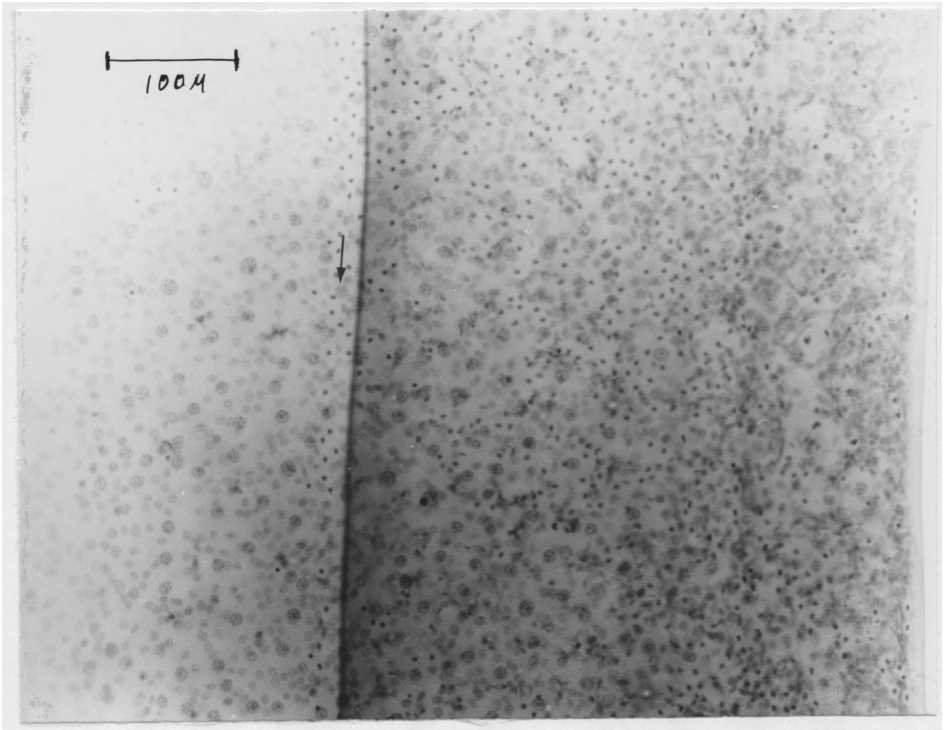


Fig. 18. Selected whole mounts of tergites of fourth instar *Blattella germanica* in which the mitoses were collected for various lengths of time by injection of Colchicine at 5 x 10^{-5} M.

a) Mitosis is beginning at the hind edge (H) of the tergite. The mitoses in the intertergite region (arrow) are associated with the differentiation of an organule. Four hour collection.

b) Mitosis has proceeded anteriorly and the first mitoses are appearing at the tergite intertergite border. Notice the organule associated division at the arrow. Eight hour collection.

c)



d)

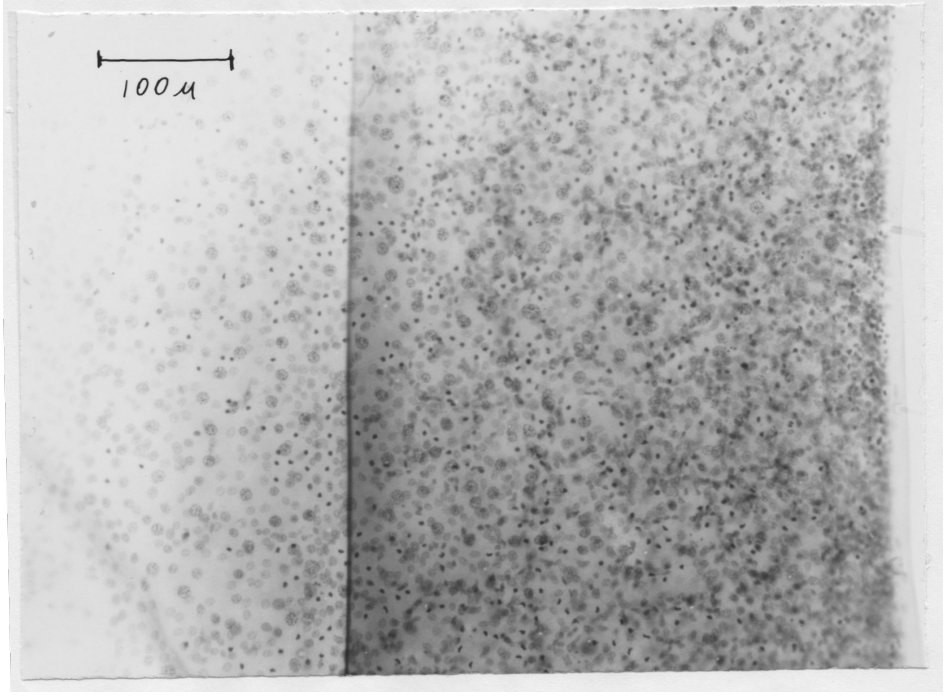


Fig. 18 cntd.,

c) A high rate of colchicine mitoses are seen at the tergite intertergite border (arrow) as well as in the mid-tergite. Yet few if any mitoses have occurred in the mid-intertergite other than differentiative type mitoses. Eight hour collection.

d) Mitoses extending over the entire tergite-intertergite. Eight hour collection.

e)

f)

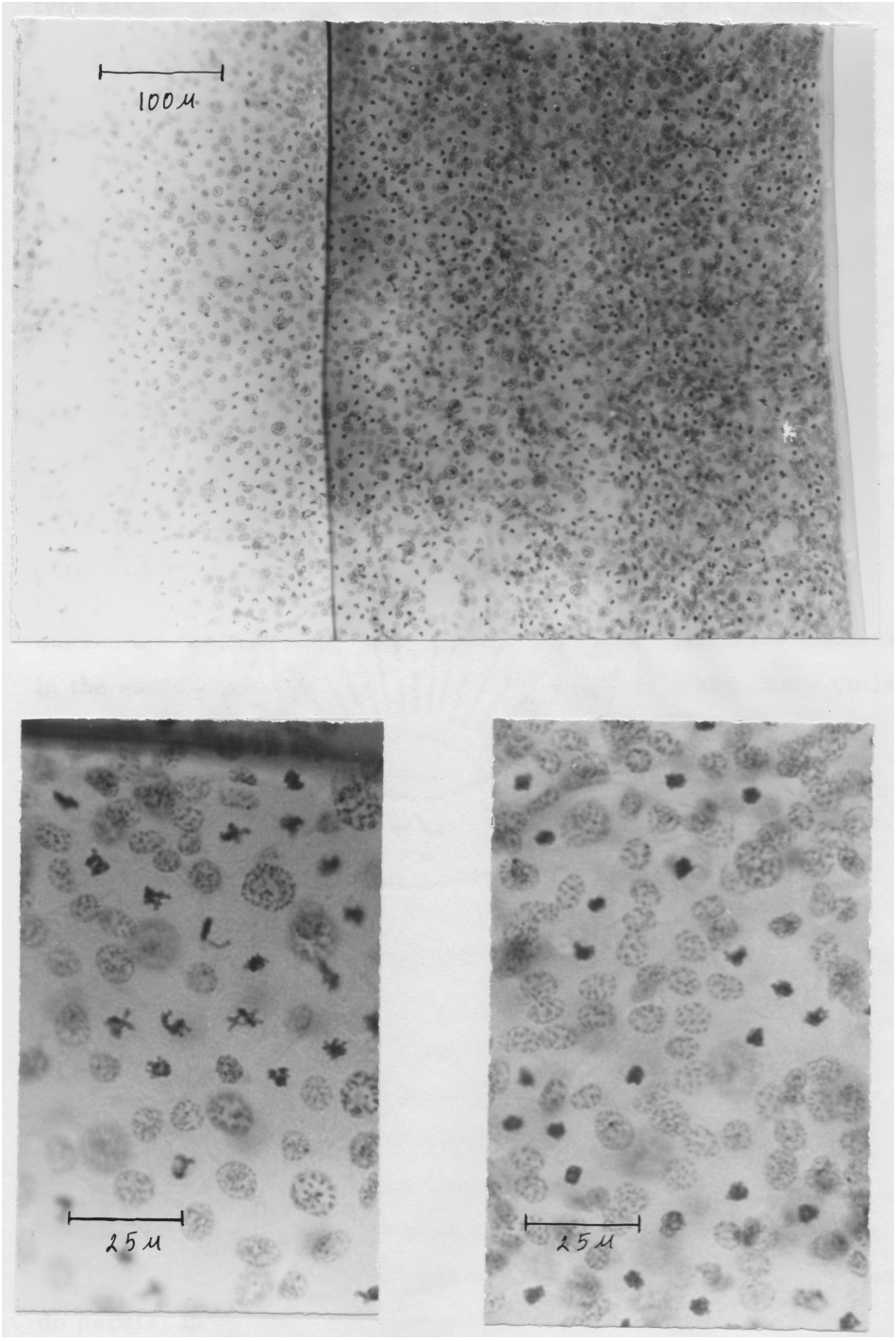


Fig. 18 cntd.,

e) An exceptional animal in which the posterior portion of the tergite shows ball mitoses, typical of high doses of colchicine, while the anterior of the tergite and intertergite show star mitoses, typical of low doses of colchicine. Presumably colchicine was high in concentration during the ball mitosis collection but was being removed by the time that the star mitoses occurred. Eight hour collection.

f) (left) Higher magnification of star mitoses.

g) (right) Higher magnification of ball mitoses.

It was previously shown that the mean time of the prothoracic gland critical period (ptg cp) occurs about the same time as the mean time of appearance of nerve coils in the molting space. In this last experiment it was shown that mitoses in the epidermis stop in the epidermis before nerve coils appear in the molting space. This suggests that the prothoracic gland is necessary to sustain molting right up to the time when the epidermal cells stop dividing and start the molting process as witnessed by the appearance of nerve coils in the molting space.

A prelude to this appearance of nerve coils in the molting space must be the detachment of at least the bristle trichogen and tormogen cells from their cuticle in order to create a molting space. This might occur simultaneously with the release of the epidermis in general from the old cuticle.

Whatever the precise first steps independent of further presence of the ptg it is of considerable interest that they occur close to these early gross morphological events of molting, the end of mitosis and appearance of nerves in the molting space.

In conclusion, this section provides the groundwork for experiments on the control of molting by establishing a sequence of events. The following experimental section attempts to establish some of the causal relationships between these events and processes which have been mapped.

D. Regeneration and the molting cycle

Regeneration in the cockroach has a profound effect upon the molting cycle and the molting cycle has a profound effect upon regeneration. This was first observed by O'Farrell and Stock (1953). As an introduction to this section I repeated their experiment using my synchronized cultures.

1. The Correlation in Time and Similar Variability of Ecdysis and the Regeneration Critical Period

The map in Figure 11 gives the T_{50} of the critical period for regeneration. Table 8 shows thatthis event is distributed about its T_{50} with a standard deviation of about 8-10 hours. An experiment was set up in which legs were autotomized from 4 samples of fourth instar NY strain nymphs at times which spanned the distribution of the event. The resulting cumulative distribution of ecdysis for these four samples is given in Figure 19 together with that for a control colony which received only the CO_2 anesthesia. Animals which have already passed the critical period for regeneration molt early without regenerating a leg. The cumulative curve then levels off while animals which have not passed the reg cp for regeneration delay their molt in order to regenerate their lost limb. The value at which the curves level off is an estimate of the proportion of the sampled population past the reg cp at the time of autotomy. In each of the cumulative curves the animals which have past their critical period for regeneration molt early compared to the mean molting time of the controls. The regeneration critical period is positively correlated with ecdysis since animals which undergo their regeneration critical period early also undergo ecdysis early. There is little variability in the timing of events between the critical period for regeneration and ecdysis, a span of about 60 hours, since any variability of important events at an ealy stage would add variability to subsequent dependent events. Figure 20 shows three distributions of the critical period for regeneration and the ecdysis of control animals. The distributions of ecdysis and the regeneration cp have remarkably similar variability. This demonstration of correlation and similar variability were only made possible by measuring the distribution of the events in time.

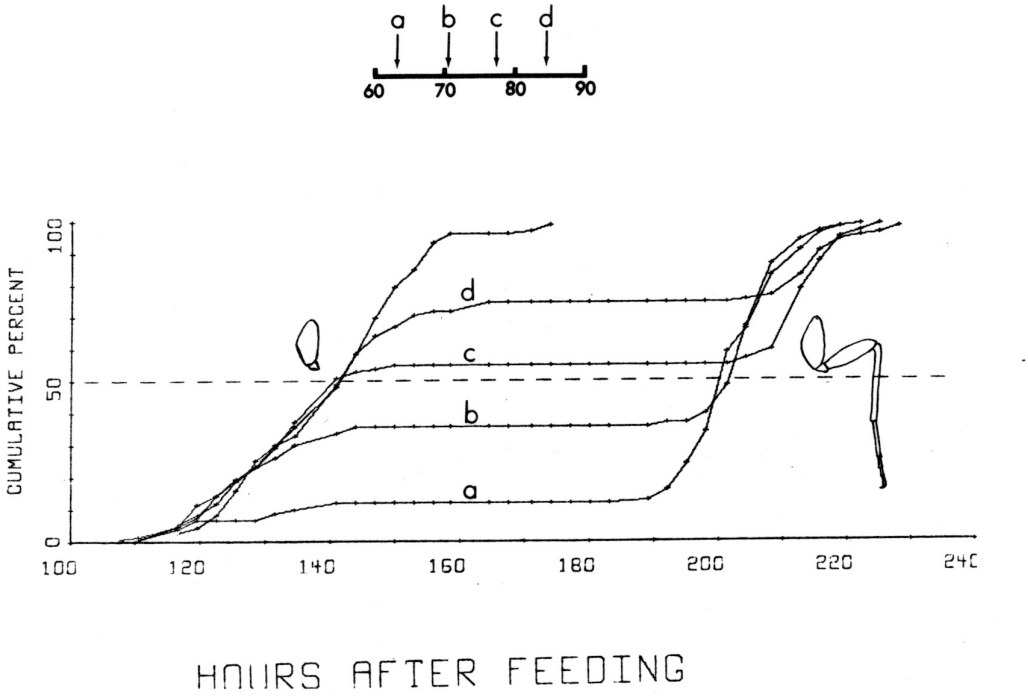


Fig. 19. Response of a culture of fourth instar *Blattella germanica* nymphs to autotomy of a limb at various times during the distribution of the critical period for regeneration. The control culture was given CO_2 anesthesia only. The experimental cultures had a limb taken off at graded intervals of time. The plateau value of the cumulative molting curve is an estimate of the proportion of animals past the critical period for regeneration at the time the leg was autotomized. Animals molting with the controls did not regenerate a limb. Animals delaying their molting cycle regenerate a complete limb.

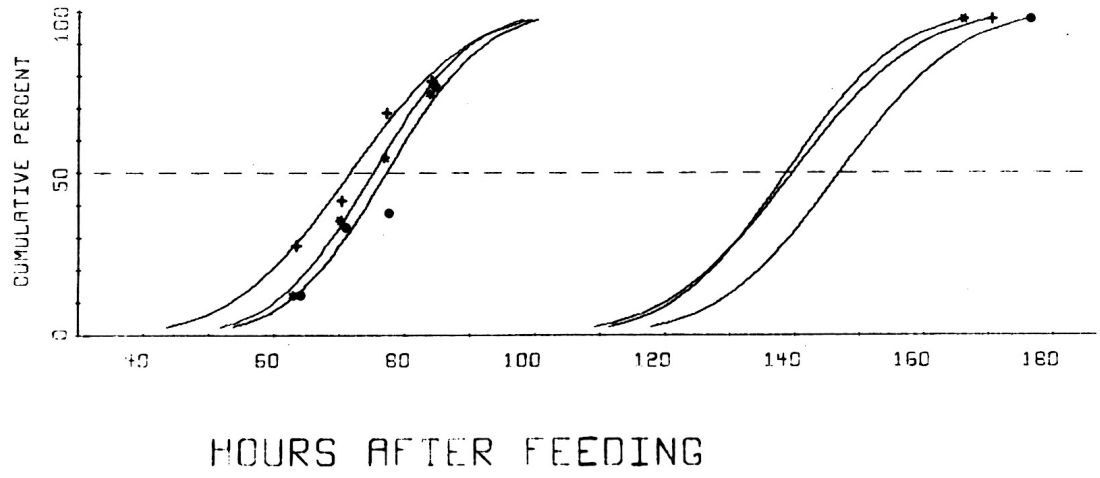


Fig. 20. The distribution of the critical period for regeneration and ecdysis. Three replicates of the experiment in Fig. 19 were performed. On the left a normal curve was fit by probit analysis to the estimates of proportions of animals past the critical period for regeneration at each autotomy time.

The estimates in Fig 20 are given by the plateau values seen for one replicate in Fig. 19. On the right a normal curve was fit to the ecdysis of the control groups for each replicate by conventional estimation of the mean and variance. Although comparison by statistical means is not possible the

distribution of ecdysis and the critical period for regeneration are virtually similar.

2. The Synchronizing Effect of Regeneration on the Molting Cycle

A synchronizing effect which regeneration has on the molting cycle was also discovered by measuring the distribution of events. This effect is visible in Figure 19. The animals which have delayed their molt to regenerate a limb have a steeper slope to the cumulative curve. This was tested more rigorously in a specific experiment using both the NY strain of *Blattella* and *Periplaneta*. The animals were synchronized by the usual method of feeding. Fourth instar nymphs of *Blattella* and sixth instar nymphs of *Periplaneta* were used. Mapping experiments had given estimates of the time when less than one percent of the animals would have passed their regeneration cp. This time was chosen for autotomizing the limbs to enable the largest number of animals to regenerate their limbs and therefor provide a maximum number of regenerating animals for comparison of the variability of ecdysis with that of controls.

Fourth instar unfed NY strain *Blattella* nymphs were randomly assigned to nine bowls. The animals were placed in the incubator and fed. At forty hours after feeding three treatments were administered. Three bowls of animals received CO_2 anesthesia only, three bowls received anesthesia plus autotomy of a metathoracic leg, and three bowls were blank controls and received neither anesthesia nor autotomy. The three bowls of CO_2 controls showed no significant difference in variability of molting from the blank controls and the 6 control bowls were therefore pooled. The results are given in Table 10. Similarly, sixth instar unfed *Periplaneta* nymphs were randomized into four bowls, fed at the appropriate time and two treatments were administered - the cockroaches in two bowls had a limb autotomized under CO_2 anesthesia and the cockroaches in the other two bowls had only CO_2 anesthesia. The results are also given in Table 10. Table 10 shows that in both *Blattella* and *Periplaneta*, regeneration of a limb causes the distribution of ecdysis to be more synchronous.

TABLE 10. --The effect of regeneration on the synchrony of molting, N=number of animals in each bowl, s^2 = the variance of molting times within each bowl.

Blattella germanica	N	s^2	Pooled variance	Variance Ratio
Controls	132	296.998		
	139	184.019		
	133	247.661		
	151	259.924		
	125	242.980		
	137	159.158		
	141	264.756		
	154	181.314		
	1112		228.77	
Regenerates	139	81.791		
	121	89.990		228.77
	125	59.148		------ = 2.860 **
	132	88.331		79.99
	517		79.99	
Periplaneta americana	N	s2	Pooled variance	
Controls	61	121.03		121.76
	44	122.79		------ = 1.491 *
	105		121.76	81.61
Regenerates	51	81.03		
	60	82.11		
	111		81.61	

* significant at 0.05 level, ** significant at 0.01 level

3. The Effect of Regeneration on Epidermal Proliferation and Other Molting Events

There were two alternatives relating the effect of regeneration to the events of the molting cycle. Either regeneration slowed down the events of the cycle and the delay of ecdysis was a result of slower progress throughout, or regeneration delayed the onset of the cycle but once started it proceeded as swiftly as the non-regenerative cycle.

These alternative hypotheses were resolved in the following experiment. A synchronous culture of fourth instar ORL strain *Blattella* nymphs were fed and at 40 hours after feeding they were randomly

split into two separate cultures - - one culture received only anesthesia while the other culture received anesthesia plus autotomy of a metathoracic leg at the femur-trochanter autotomy point. Each culture was subsequently sampled at intervals, 20 animals per sample, and the animals abdominal tergites prepared as whole mounts with Feulgen staining of their nuclei. The nuclear density of the epidermis in the inter-tergite region was measured in a random sample of 5 animals from each sample of 20. The results are shown in Fig 21. No regression lines were fitted to the data. Since a previous study had shown that the data would not be normally distributed, but there are clear differences even in the raw data. The samples of 20 whole mounts were also scored for the appearance of nerve coils in the molting space. Ecdysis was also followed in the controls and regenerating culture. Probit analysis was performed on this data (Table 11).

TABLE 11.- -The effect of autotomy on the T_{50} of nerve coil appearance and ecdysis in fourth instar *Blattella germanica*

						Tests of Significance (p)		
Event	N	Nw	T_{50}	Sx	St_{50}	Normality	Paralellism	Sequence
Nerve coils:								
controls-CO_2	102	46	72.5	8.8	1.3	$p > 0.1$		
							$p > 0.05$	$p < 0.01$
autotomized	160	59	117.3	11.6	l.5	$p > 0.3$		
Ecdysis:								
controls	126	-	124.2	11.6	1.0	$p > 0.1$		
							-	$p < 0.01$
autotomized	120	-	170.0	8.0	0.7	$p > 0.1$		

p = probability that there is no significant difference

Mean delay of nerve coil appearance = 44. 8 +/- 4.0 hrs.

Mean delay of ecdysis = 45.8 +/- 2.4 hrs.

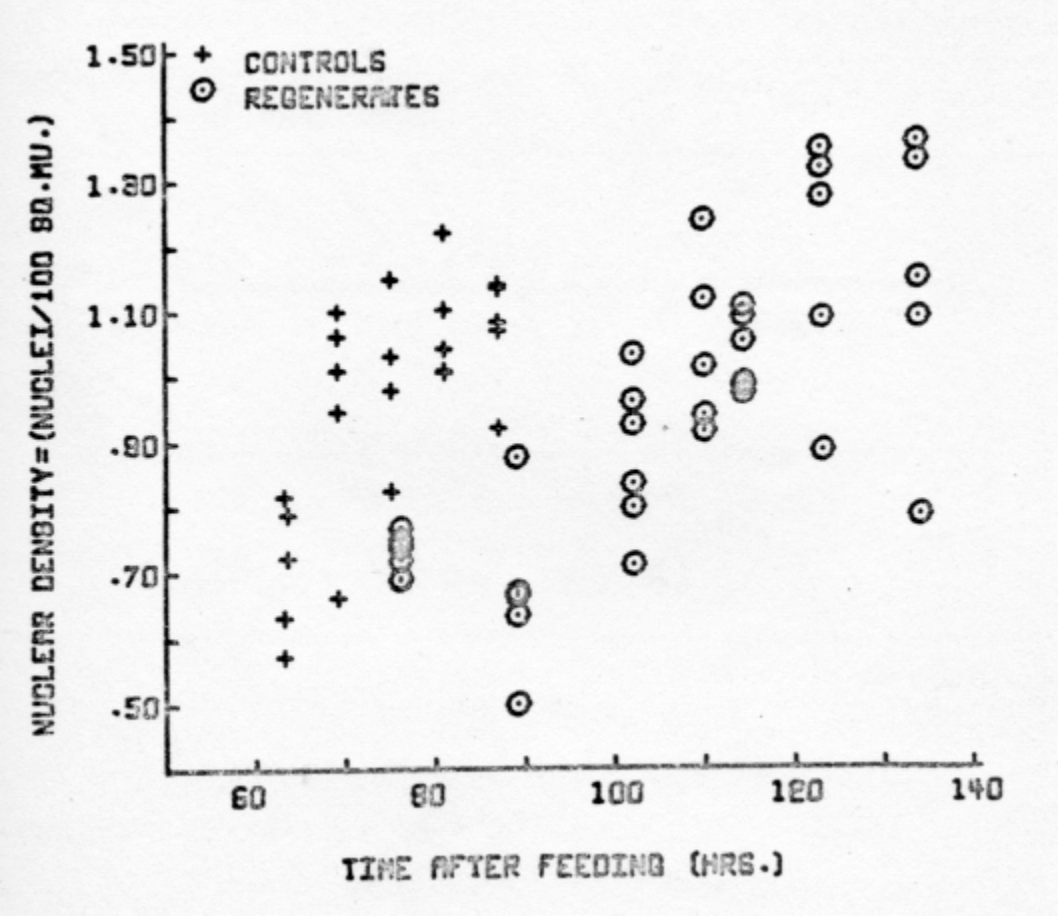


Figure 21. The effect of regeneration on epidermal proliferation in IV instar *Blattella germanica* nymphs as measured by the nuclear density of the intertergite region. A synchronous culture was split in two and one of the subcultures served as a control receiving only CO_2 anesthesia. The other culture had a metathoracic leg removed at the trochanter-femoral autotomy point.

The results of this experiment confirm the hypothesis that regeneration causes a delay in the onset of the molting cycle. Both the appearance of nerve coils and ecdysis were delayed a comparable amount which implies that the delay is not just a slowing down of the molting process but a delay in its inception. It is notable that the proliferation of the epidermis was delayed also.

In animals which had not yet passed their critical period, O'Farrell and Stock (1954) showed that autotomy causes a new critical period for regeneration to be induced. That is, it extended the period when regeneration could occur if another leg was autotomized subsequent to the first. I measured this delay of the regeneration c.p. and as in the above experiment the amount of delay was comparable to the delay in ecdysis.

These experiments demonstrate the interaction between regeneration and the molting cycle. The feedback of regeneration upon molting occurs on some event prior to epidermal proliferation. Since regeneration also causes a delay of the regeneration cp, it is possible that the regeneration feeds back on the brain to prevent the last brain function necessary for inducing a molt. The possibility that this feedback is mediated via the nervous system is considered in section F.

E. Quantitative Aspects of Regeneration

So far in this study regeneration has been considered as an all-or-none phenomenon. While this is true in the sense that autotomy results either in a functionally complete regenerate or no regenerate at all, it is not true as far as the size of the regenerate is concerned. Pohley (1966), working with regeneration of wing discs in the meal moth *Ephestia*, demonstrated a correlation between the size of the regenerate and the length of delay of molting. Also both O'Farrell and Stock (1954) working on *Blattella* and Pohley working on *Ephestia*, have shown a slightly greater delay of molting associated with regeneration of two rather than one disc or limb, that is, a positive correlation of delay with number of limbs. Both of these authors hypothesize from these correlations of amount of tissue with delay that the delay is caused by the amount of tissue regenerating. I set out to test this hypothesis which up to now is based purely on correlation rather than causal evidence.

Before doing this, some preliminary work on the course of regeneration was necessary.

1. Continued Regeneration of a Limb After the Initial Regeneration

The regenerated limb has been shown to be different from the original limb in a number of ways. Figure 22 shows a normal limb and a regenerate one molt after autotomy. The regenerate is noticeably smaller than the normal leg. Brindley (1897) demonstrated that the regenerated limb has four tarsal segments as opposed to the normal five. This has been shown to be true for the regenerated tarsi of all orthopteroid insects (Bordage, 1905). In addition, Penzlin (1963) has shown that certain gross differences in the tracheal supply occur when a leg is regenerated. The primary change is in the femur which has a greatly reduced tracheal trunk system. However the tarsus of the regenerate has a more extensive trunk supply than the normal. Since these characteristics of the regenerated limb differed from the normal limb, a series of other characters of the limbs were chosen which were easily quantified. These were followed in both the normal leg and the regenerated leg through a series of molt cycles. A metathoracic leg was autotomized at the trochanter-femoral autotomy point from each of 500 unfed first instar NY strain *Blattella* nymphs. A sample of 20 animals was taken during each molting cycle and the following characters were scored for both the regenerated limb and its contralateral control limb:

1. Length of the first tarsal segment in microscope ocular units.

2. Number of bristles on the first tarsal segment.

3. Number of femoral spines.

4. Number of tibial spines.

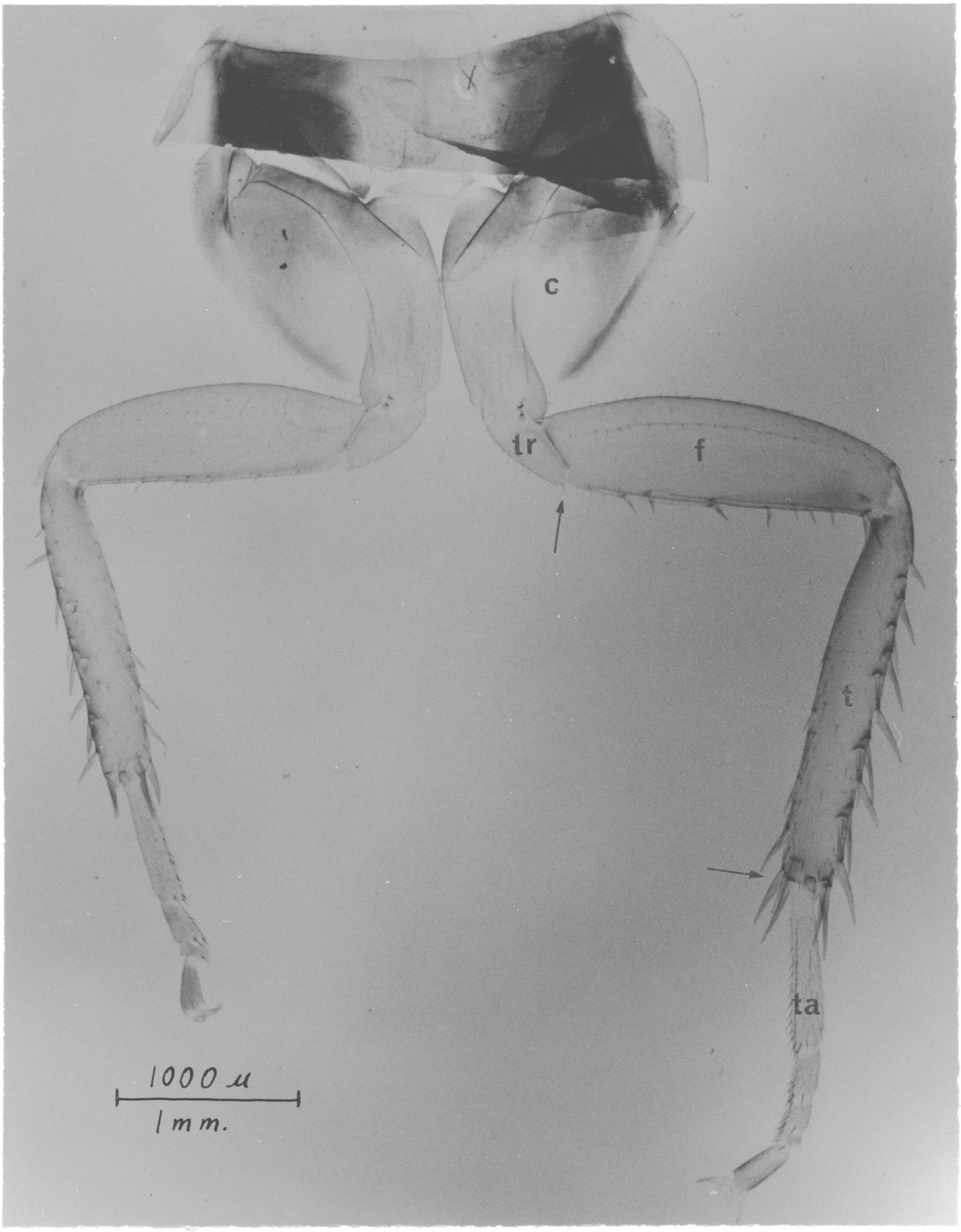


Fig. 22. A normal and regenerate limb showing segments and autotomy points (arrows). Coxa(c), trochanter (tr), femur (f), tibia (t), tarsus (ta).

TABLE 12--Long term regeneration of limbs autotomised in the first instar

Character	Instar	Calculated Means Regenerate(R)	Control(C)	C-R=d	t_{obs}
1st tarsal length	I		245.6		
(ocular units)	II	238.8	321.6	+82.7	+23.106*
	III	384.9	434.1	+49.15	+17.15*
	IV	501.3	533.8	+32.45	+5.12*
	V	663.0	668.1	+5.1	+2.51
	VI	890.9	887.7	-3.2	-0.731
	adult	1152.8	1139.1	-13.7	-2.06
1st tarsal	I		49.9		
bristle no.	II	31.27	53.66	+22.4	+27.53*
	III	50.2	71.2	+21.0	+13.39*
	IV	80.1	84.8	+4.7	+2.065
	V	110.8	105.9	-4.95	-3.52 *
	VI	130.9	126.9	-3.95	-2.01
	adult	157.2	149.0	-8.2	-4.27 *
femoral spine no.	I		1		
	II	1.44	1.94	+0.5	+4.123*
	III	3.05	3.35	+0.3	+1.831
	IV	8.45	6.35	-2.1	-4.64 *
	V	11.35	10.25	-1.1	-3.39 *
	VI	13.15	11.15	-2.0	-4.727*
	adult	13.85	11.20	-2.7	-6.012*
tibial spine no.	I		2		
	II	6.0	15.22	+9.22	+24.56*
	III	18.55	20.15	+1.60	+4.56*
	IV	22.90	23.00	+0.10	+0.252
	V	23.9	24.05	+0.95	+2.16
	VI	26.0	26.1	+0.10	+0.208
	adult	26.2	26.6	+0.35	+0.662

* character-wise significant at 0.05 level

Table 12 gives the means for the collected data. The important column to follow is the far right column of figures, t_{obs}, for each character. A t_{obs} is student's t measure of significant difference and is given by the formula.:

$$t_{obs} = d/(s^2/n)^{1/2}$$

where d =the mean difference.
s = standard deviation of the difference
n = sample number

T-critical is defined as the border between significant and insignificant difference with 5% confidence (t=2.101). This allows for 5% of the differences to be significant by chance. However, a character-wise confidence of 5% can be obtained which allows a 5% probability of a single mean difference for a given character being significant. This character-wise confidence is given by a t-critical of 2.86. When t_{obs} is greater than 2.86, the control value is significantly larger than the regenerate; when t_{obs} is less than -2.86 the control values are significantly smaller than the regenerate; when t_{obs} is between -2.86 and +2.86 no significant difference can be detected at the 5% level.

A character-wise instead of experiment-wise significance level had to be used in this experiment because it could not be assumed that the differences of one character are independent of the differences of each other character. As a result the conclusions for a given character are not statistically independent of the conclusions for the other characters. For example one might expect a larger tarsal segment to have more bristles on it. As a result if one detected a difference in length between the regenerate and control tarsal when no real mean difference exists, then one might also detect a mean difference in bristle number, compounding the error. In light of this,Table 12 must be read for each individual character. No comparisons should be made between characters within an instar.

If the regenerating limbs were approaching the control limbs as a limit one would expect an initial difference between the control and the regenerates in the first instars after regeneration with a gradual disappearance as the regenerated limb approaches the control limb. This is observed in the case of tibial spine number in Table 12. But the other characters - - tarsal length, tarsal bristle number, and tibial spine number -- do not approach the control as a limit. All three end up with larger values than the controls. It is somewhat understandable that tarsal length and tarsal bristle number end up greater than the controls since the regenerated tarsus has only 4 segments compared to the control's 5. It might be expected that the individual tarsals of the tarsus would be longer to compensate for their fewer number .

The regenerated femur and tibia are harder to measure but these measurements were performed on the samples of 20 adults of Table 12, and they were found not to be significantly different from the controls. It is surprising that the femoral spines of the regenerate consistently outnumber the spines of the control femur. It is interesting that both tarsal length and bristle number approach their limits slowly while, femoral and tibial spine number have essentially reached an equilibrium by the third molt. Tibial spine number approaches the number of spines in the control but femoral spine number approaches a greater number than the control leg.

This difference in femoral spine number compounds the already known differences between the regenerate and the control, tracheation and tarsal number. The demonstration of tarsal length and bristle number differences are not surprising considering the already known difference in tarsal number. These differences emphasize the fact that in some respects, the regenerated limb is not a replica of the original limb.

2. Short Term Regeneration of the Limb

The amount of regeneration has been shown to be directly correlated with the amount of delay of the molting cycle (Pohley, 1966, Penzlin, 1963). While corroborating this I have also shown that the amount of delay and amount of regeneration are also dependent upon when the amputation occurred.

Figure 23 shows the amount of regeneration and the time of ecdysis for groups of sixth instar ORL strain nymphs which had a leg autotomized at different times after feeding. The sixth instar nymphs are useful because their critical period for regeneration occurs at about 110 hours after feeding compared to 65 hours for the fourth instar. This provides a larger span of time for observing the effect of time of autotomy on regeneration. An inset in figure 23 shows that the length of time from autotomy to ecdysis can be explained by a third degree polynomial in regenerated tarsal length. This implies that the volume or mass of the regenerate is linearly related to the time between autotomy and ecdysis. This is not surprising since this is merely a more refined way of stating Penzlin's and Pohley's results. In addition O'Farrell and Stock (1953) as well as Brindley (1897) showed that regeneration of the tarsus causes less delay than regeneration of the femur-tibia-tarsus. Thus there are three types of evidence correlating amount of regeneration with amount of delay:

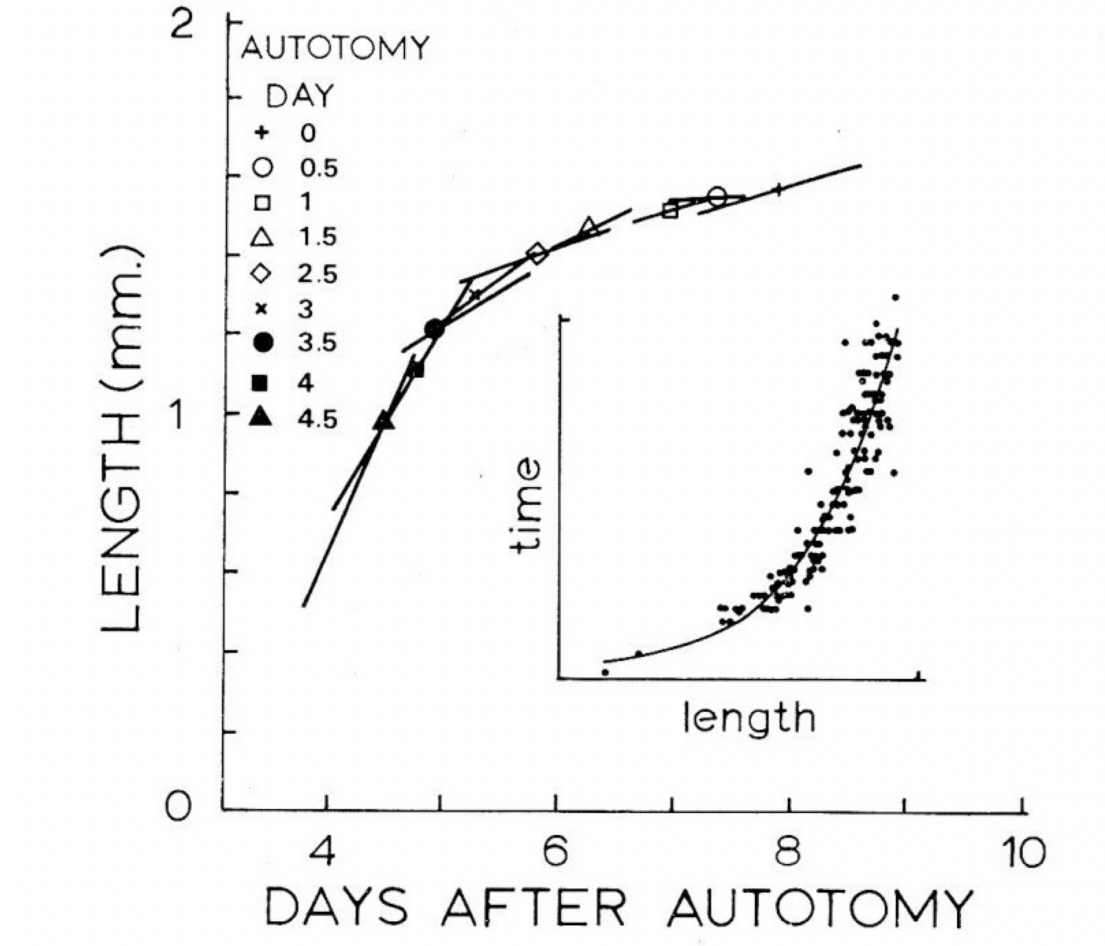


Fig. 23. The relationship of the length of the regenerated tarsus to the time from autotomy to ecdysis in *Blattella germanica*. To obtain varying times for autotomy to ecdysis the time of autotomy was varied as indicated.

1. Tarsal regeneration delays molting less than femur-tibia-tarsal regeneration.

2. The volume or mass of regeneration of a particular part is directly correlated with the amount of delay that had occurred in that specific animal.

3. Regeneration of two legs delays ecdysis longer than regeneration of a single limb.

The next experiments were designed to establish the causal relationship between these two correlated variables, amount of regeneration and delay of ecdysis; does an extra delay cause extra regeneration, or does extra regeneration cause extra delay?

3. The Effect of the Size of the Autotomized Leg on the Amount of Tarsal Regeneration

In order to obtain animals which had strikingly different sized legs, I took a colony of one thousand third instar nymphs ONY(l:3), one half orange body phenotype and one half wild type phenotype, and autotomized a metathoracic limb from each. These animals were allowed to regenerate their limb, which resulted in fourth instar nymphs with one normal-sized metathoracic leg and one relatively small regenerated metathoracic leg. I then asked the question: Would taking off a tarsus from a small leg result in a significantly smaller regenerated leg at the next molt than would the removal of a tarsus from a normal leg?

To control for bowl effect I used the genetic marker orange body so that both treatment groups, small-leg autotomy and normal-leg autotomy, could be raised in the same bowl. To control for the possibility of differences between the phenotypes, a replication of the experiment was run in a separate bowl in which the autotomy treatments assigned to each phenotype were reversed. It was assumed in the design of the experiment that there is no bowl-phenotype interaction. The animals were assigned to their bowls randomly, fed and at 40 hours after feeding a leg was removed. from each animal.
The time of autotomy was completely confounded with the bowl effect. The results given in Table 13 demonstrate that a smaller autotomized leg will result in a smaller regenerate. This experiment sets up the design for the next question: Does the smaller regenerate result in a shorter delay of the molting cycle?

4. The Effect of the Size of the Autotomized Leg on the Delay Due to Tarsal Regeneration

This experiment was set up exactly as the previous experiment except that instead of following the size of the regenerate, the amount of delay caused by regeneration was followed. The amount of difference of regenerate length observed in the previous experiment, considering the fact that delay is a third degree polynomial in regenerate length, should be enough to cause a sizeable difference in delay between the two different treatments. However Table 14 points out that there is no significant difference in molting time for an animal regenerating a small tarsus as compared to a normal-sized tarsus. This demonstrates that the amount of delay is not caused by the amount of the regenerating tissue. Instead, the size of the regenerate depends upon the amount of delay.

5. The Effect of Double Autotomy on the Lengths of Delay of the Molting Cycle

Since the amount of tissue was not the controlling factor in the length of delay, I reexamined the experiments on simultaneous double autotomy performed by Pohley on *Ephestia* and O'Farrell and Stock on *Blattella.* However, I used the refined techniques of comparison and controls developed in the above experiments.

The goal was to test the effect of the number of legs amputated, single or double, as well as the level of autotomy, tarsal or femur-tibia-tarsal. If the amount of tissue were important in controlling the

delay, there should be a greater additional delay due to a second femur-tibia-tarsal autotomy than in a second autotomy of the tarsus only. This would show up as a level-number interaction in the experiment.

TABLE 13.--Effect of size of the autotomized limb on the amount of tarsal regeneration

Equation to be fit: $Y_{ijkl} = U + B_i + P_j + S_k + e_{ijkl}$

Y_{ijkl} = the length of the regenerated first tarsal segment of $ijkl^{th}$ animal
U = a grand mean of tarsal length
B_i = effect of bowl i on tarsal length, i =1, 2
P_j = effect of the phenotype on tarsal length, j=1, 2
S_k = effect of size of the autotomized limb on tarsal length
e_{ijkl} = error associated with $ijkl^{th}$ animal

ANOVA

Source	D. F.	Sums of squares	Mean square	F ratio
Bowl effect(B_i)	1	3.37	3.37	.2553
Phenotype effect (P_j)	1	.42	.42	.0318
Size effect (S_k)	1	2,876.46	2,876.46	217.9774**
Residual	220	2,903.15	13.20	
Total	223	5,783.40		

** significant at .01 level

TABLE 14. --Effect of size of the autotomized limb on the amount of delay of the molting cycle

Equation to be fit: $Y_{ijkl} = U + B_i + P_j + S_k + e_{ijkl}$

Y_{ijkl} = time of ecdysis of $ijkl^{th}$ animal
U = a grand mean of ecdysis times
B_i = effect of bowl i on time of ecdysis, i=1, 2
P_j = effect of phenotype on time of ecdysis, j=1, 2
S_k = effect of size of autotomized limb on time of ecdysis, k=1, 2
e_{ijkl} = error associated with $ijkl^{th}$ animal

ANOVA

Source	D.F.	Sums of squares	Mean square	F ratio
Bowl effect (B_i)	1	136.5	136.5000	2.1503
Phenotype effect (P_j)	1	74.8	74.8000	1.1785
Size effect (S_k)	1	152.0	152.0000	2.3444
Residual	236	14,981.6	63.4813	
Total	239	15,344.9		

The experiment is complicated by the need for the genetic marker orange body to allow two contrasting treatments to be run in the same bowl, making it necessary to measure the phenotype effect. In addition, row and column effects had to be measured since four bowls were used in two rows and two columns. In the complete design it was assumed that the interactions of the rows and columns with the other main effects were negligible. As it stood, the design called for estimating a mean, a row effect, a column effect, a phenotype effect, a level of autotomy effect, a number of autotomy's effect and a level-number interaction. This represents 7 effects to be estimated. Since another estimate would give a complete parameterization of the design, the phenotype-level interaction was measured.
In all, 832 animals were used. They were randomly selected from a colony of 2000 unfed ONY (1:3)

fourth instar nymphs and assigned to the four bowls in groups of 10. Equal numbers of orange body and wild type phenotypes were maintained in each bowl. They were placed in the incubator, left to equilibrate for 12 hours, and then fed. After 40 hours with food, the leg autotomy was carried out. In each bowl single and double autotomy were carried out simultaneously. Each bowl of 200 animals was completed within a half hour and the small differences in autotomy time were completely confounded with the row and column effects.

The analysis of the results of the experiment are given in Table 15. The level and number effects are significant. There is no significant level-number interaction, which indicates that both levels of autotomy show the same response to a second autotomy. This is additional proof that the amount of delay caused by regeneration is not caused by the amount of tissue regenerated. The raw data allows the observation that the double-autotomy animals have a different distribution of ecdysis from the single-autotomy animals. Figure 24 shows the cumulative curves of ecdysis for one of the four bowls of animals in the experiment of Table 15. This particular bowl was a comparison of single and double autotomy at the level of the trochanter-femur autotomy point. It it typical of the results seen in the other three bowls. The single-autotomy curve is a typical sigmoid curve indistinguishable from a normal distribution ($p > 0.1$). But the double-autotomy cumulative curve is not compatible with a normal distibution ($p < 0.01$). It starts out very slowly, then increases very rapidly and stops abruptly. In looking at the data of Figure 24 it is easy to see a relation between the two curves, but this data was used only as a suggestion for a hypothesis to be tested in a follow-up experiment. The curve for double-autotomy is the square of the curve for single-autotomy. This suggests that the feedback of each regenerating limb is independent of every other regenerating limb in the animal. As a result, the feedback from each limb must stop before the molting cycle can ensue. Feedback is independent for each limb. Thus when half of the total number of limbs of a colony of animals with double autotorny have ceased their feedback, only $1/2 \times 1/2 = 1/4$ of the animals will have ceased feedback in both limbs. Figure 24 also gives the squares of the values for single-autotomy ecdyses. They lie remarkably close to the actual observed values of the double-autotomy ecdyses.

The slight extra delay (5.38+/- 68 hrs.) of double autotomy over single autotomy can be accounted for by independent feedback from the regenerating limbs and not by the extra mass of tissue regenerating. This is instead a consequence of the extra, delay associated with the necessity of both feedbacks stopping before molting can proceed.

TABLE 15.--The effect of double autotomy and level of autotomy on the time of ecdysis

Equation to be fit: $Y_{ijklmn}=U+R_i+K_j+P_k+N_l+L_m+NL_{lm}+LP_{mk}+e_{ijklmn}$

Y_{ijklmn} =time of ecdysis of ijklmnth animal
U =grand mean of ecdysis times
R_i =row effect, i=1, 2
K_j =column effect, j=1, 2
P_k =phenotype effect, k=1, 2
N_l =effect of number of legs autotomized, l=1, 2
L_m =effect of level of autotomy, m=1, 2=femor, tarsal
NL_{lm} =interaction between number and level of autotomy
LP_{mk} =interaction between level and phenotype
e_{ijklmn} =error associated with ijklmnth animal

ANOVA

Source	D. F.	Sums of squares	Mean square	F ratio
Row effect (R_i)	1	5,285.35	5,285.35	98.17**
Column effect (K_j)	1	2,736.88	2,736.88	50.83**
Phenotype effect (P_k)	1	518.80	518.80	9.63**
Number effect (N_l)	1	6,036.15	6,036.15	112.12**
Level effect(L_m)	1	146,412.67	146,412.67	2,719.64**
Number-Level Interaction(NL_{lm})	1	3.90	3.90	.07
Level-Phenotype Interaction(LP_{mk})	1	21.90	21.90	.41
Residual	824	44,360.26	53.84	
Total	831	205,375.91		

Mean difference due to leg number =
$N_2 - N_1 = 5.38 \pm .68$ hrs.

Mean difference due to level of autotomy =
$L_2 - L_1 = 26.53 \pm .68$ hrs.

**significant at .01 level

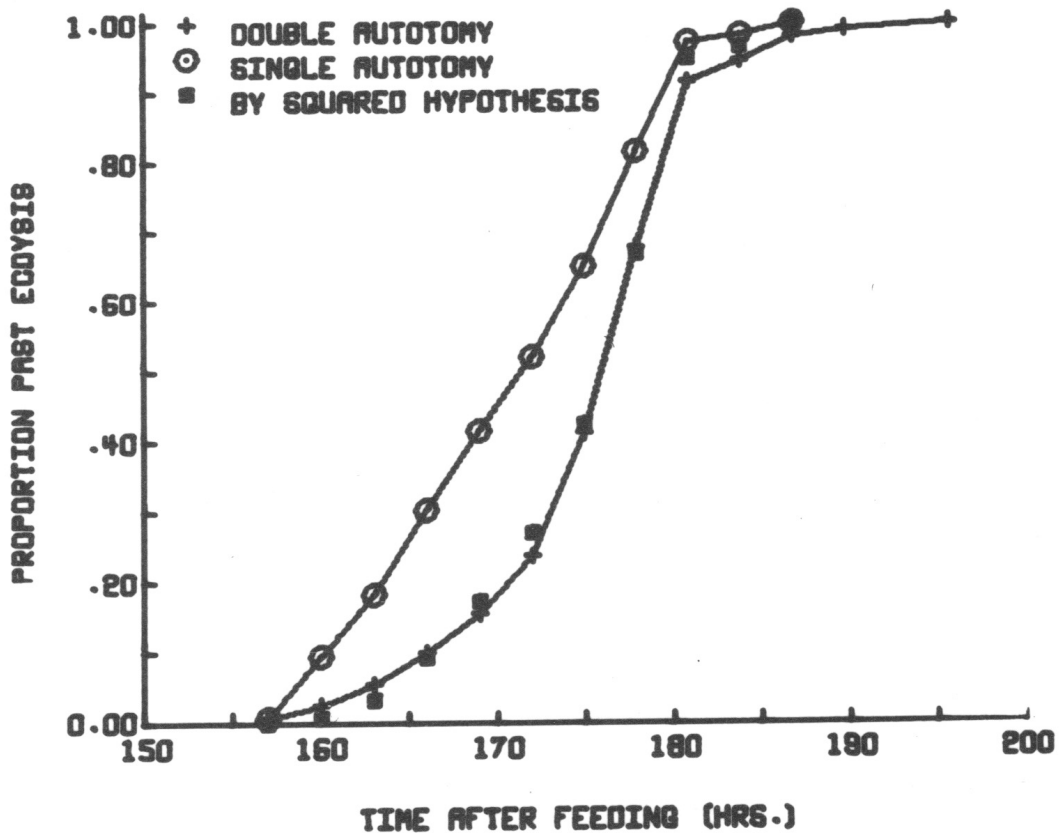


Fig. 24. The effect of double as compared to single autotomy on the time of ecdysis in fourth instar *Blattella germanica* nymphs. Also plotted are the predicted times of ecdysis (squares) for double autotomy if the feedback of regeneration on the delay of molting is independent for each leg. (explained in text)

F. Nerves and the Control of Molting and Regeneration

Although it is possible to envision humoral feedback working in the above manner, there already exists a mechanism by which the above feedback could work via the nervous system. It was previously shown that the head critical period and the regeneration critical period are indistinguishable. This suggests that the feedback of regeneration on the molting cycle may alter the brain's initiation of the molting cycle. If the brain were "aware" that a leg was amputated and it hadn't already finished its role in initiating a molting cycle, it might be able to delay the initiating steps in order to provide time for limb regeneration. Wilson (1966) has already shown that the cockroach is "aware" that it has lost a leg since it changes its walking pattern when a leg is amputated. Pringle (1956) has described leg proprioceptors in *Periplaneta.* I have found a similar distribution of proprioceptors in *Blattella*. The positioning of these proprioceptors would enable a roach to differentiate between a tarsal autotomy and

a trochanter-femoral autotomy. Thus elements necessary for a nervous mechanism are present.

This experimental section describes some experiments testing the role of nerves in the control of molting and regeneration.

1. Amputation at non-autotomy points v.s. autotomy points.

Penzlin (1963) noted that amputation at non-autotomy points results in smaller regenerates than amputation at the autotomy points. His experiments were performed on *Periplaneta americana* nymphs just after an ecdysis to ensure enough time for regeneration to occur. I repeated his experiments with amputations performed closer to the critical period for regeneration. Thus the time of amputation was known to be within the distribution of the critical period for regeneration of the culture. Amputations at the atypical points and those at the autotomy points are compared in relation to the critical period for regeneration. The following are the types of amputation performed:

Two controls :

1. controls - CO_2 anesthesia only

2. unilateral cercal amputation-injury control

Four leg amputations listed in distal to proximal order:

3. tarsal autotomy

4. amputation at the femoral-tibial joint

5. femoral autotomy

6. amputation through the distal third of the coxa

Figure 25 shows the cumulative percent which completed ecdysis. The CO_2 controls and the injury control give relatively smooth sigmoid cumulative curves. The two autotomys give the typical early non-regenerates, followed by a plateau, followed by the ecdyses of the regenerated animals. Judging from the plateau, the operation was perform ed when 15% of the animals had passed their critical period for regeneration. Amputations at both atypical positions gave different cumulative patterns of ecdysis. The amputation at the femoral-tibial joint, called tibial amputation in Figure 25, would have been expected to give a longer delay than tarsal autotomy but a shorter delay than femoral autotomy. However, tibial amputation caused an even greater delay for some animals than femoral autotomy. The tibial amputation curve does not plateau and non-regenerates appear up to 170 hours after feeding. Tibial amputation did result in progressive partial to complete regeneration from 170 hours on. A clue to the cause of this atypical cumulative curve is given by examining some of the non-regenerates from 130 to 170 hours. Many of these non-regenerates lost their femur though it was not removed at the amputation time. It is possible that these animals had autotomized the stump of the femur subsequent to the operation and having passed their critical period for regeneration, could not delay the molting cycle. Thus they molted without regenerating. Bohn (1964) has also observed autotomy of a stump subsequent to amputation at an atypical point in the African roach, *Leucophaea maderae*, although he has also shown that the roach can and does start regenerating limbs at atypical points.

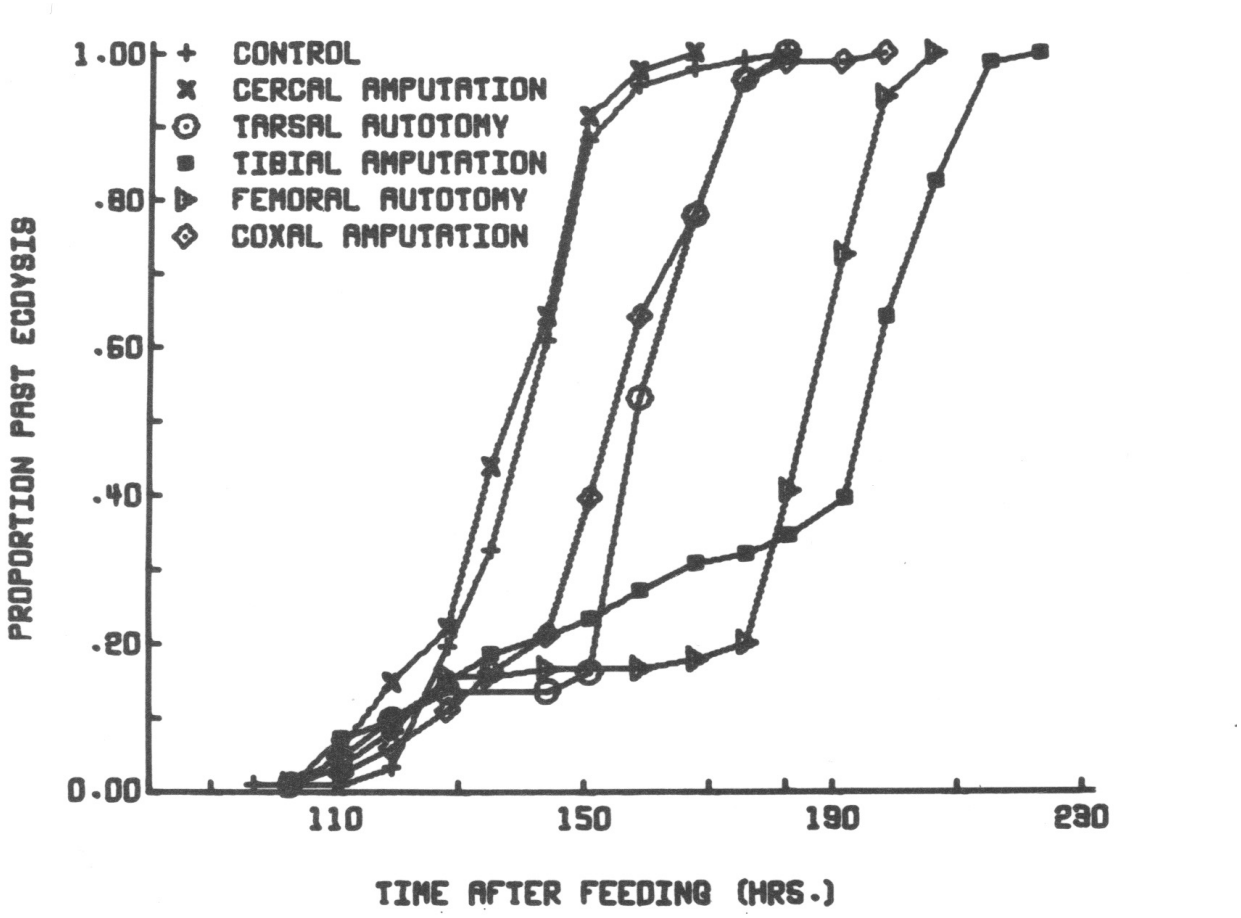


Fig. 25. The effect of amputation of an appendage at non-autotomy points on the pattern of ecdysis in groups of about 60 fourth instar *Blattella germanica* nymphs. The CO_2 control and the unilateral cercal amputation molt without any delay of the molting cycle. animals amputated at the traditional autotomy points show the plateauing effect of the cumulative curve separating the non-regenerates from the regenerates. The plateau values indicate that the experiment was performed when approximately 15% of the animals were past the regeneration critical period. The curves for the tibial and coxal amputation are discussed in the text.

Thus it seems possible arguing from data in Figure 25 that amputation at an atypical point could not delay the molting cycle, but the expected delay could be induced by subsequent autotomy of the stump. This seems reasonable if a nervous mechanism is involved. Insects are typified by stereotyped movement and responses, and the limited avenues of sensory input are often given as the cause. Loss of a leg in nature most likely occurs at the autotomy points. (Only a slight tug by the experimenter on the animal can result in the autotomy, whereas other joints have to be cut in order to be separated.) Therefore the animal's sensory apparatus for delaying a molt might not be activated by amputation at the atypical points. If the stump were subsequently autotomized through reflex preening, the animal would be able to gather sensory information from the autotomy process and could delay molting. This could explain the extra delay observed in the tibial amputees in Figure 25.

The coxal amputation of Figure25 also show an atypical cumulative ecdysis curve. There is a definite delay of ecdysis of the coxal amputated animals but it is not the expected delay that would

accompany regeneration of such a large amount of tissue. Like tibial amputation, the coxa involves an atypical amputation point and although regeneration can and does occur from a cut coxal surface, it is not functionally capable of delaying the molting cycle. The slight delay mentioned above, is on the order of the delay seen in tarsal regeneration. It may be due to the more drastic nature of coxal amputation in which a greater amount of blood is lost than in any of the other amputations.

2. The Effect of Section of Leg Nerves on the Delay of Molting and Regeneration

Direct evidence was sought for the implication of nerves in the delay of molting. The first experiment along this line was to compare the sectioning of the nerves to the leg with autotomy of a limb. Sixth instar nymphs of *Periplaneta americana* were used for these experiments because their large size made the nerve sectioning easier. Many of the phenomena concerning regeneration in *Blattella* have been confirmed in *Periplaneta*.

In the first experiment, four treatments were given:

1. Control - CO_2 anesthesia, only

2. Sham operation - the metathoracic sternite was cut at the position that the nerves were cut.

3. The nerves from the metathoracic ganglion to one of the metathoracic legs were cut with iridectorny scissors. Success of the nerve section was tested by observing its use in walking and by testing the irritability of the leg.

4. A metathoracic leg was autotomized at the trochanter-femoral autotomy point.

These four treatments were administered to synchronous colonies of sixth instar *Periplaneta* nymphs at two different times. The first time was 84 hours after feeding and the second time 98 hours after feeding. These two times were respectively 5 and 20% past the critical period for regeneration.

The third treatment group gave unexpected results for both treatment times. Most of the animals of treatment three with cut nerves spontaneously autotomized their limbs within the next 24 hours.

A few (17 out of 115) of these nerve-sectioned animals delayed their molting cycle long enough to succeed in partially regenerating a leg but they were non-functional and non-irritable legs, judging from their behavior.

These results were inconclusive, but they pointed to a possible necessity of nerves for regenerating limbs.

3. The Timing of Nerve Section and Regeneration

If nerve section were performed after the critical period for regeneration, the leg could not regenerate. However a question worth asking is: Would additional time enable a leg to regenerate in a nerve-sectioned animal? Two experiments were performed to answer this question. In one experiment time of nerve section was varied. The treatments were given to the randomly selected groups of individuals at 22, 42, 68, and 92 hours after feeding. Two types of treatment were given:

1. autotomy of a metathoracic leg at the trochanter-femoral autotomy point

2. section of the nerves to a metathoracic leg plus autotomy at the trochanter-femoral autotomy point

The number of regenerates and non-regenerates for each treatment are given in Table 16:

Table 16

Time of Operation (Hrs. after feeding)	Autotomy only		Autotomy Plus Nerve Section	
	Non-Reg.	Reg.	Non-Reg.	Reg.
22	0	21	8	14
42	0	25	12	13
68	0	23	14	18
92	8	17	16	3

It is noticed at once that the critical period distribution is not reached until after 68 hours. This is consistent with results of past experiments on *Periplaneta*. The question to be asked is whether the time before the critical period had any effect on the proportion of animals regenerating a limb in the 22, 42, and 48 hour animals. If time were the determining factor, a contingency test should show that the number of animals regenerating is not independent of time. But there is no significant effect of time on the proportion of nerve-section regenerates. (Chi Square, 2 df = 0.8 ,$p > 0.4$)

Another test of whether time is an important factor in controlling the number of nerve sectioned animals regenerating a leg was given by the following experiment:

Four treatments were given to Seventh instar *Periplaneta* nymphs:

1. control - sham operation
2. autotomy of a leg
3. autotomy of a leg, plus section of its nerves
4. autotomy of a leg, plus section of its nerves, plus autotomy of the contralateral leg.

If nerve-sectioned legs were not regenerating because they lacked the time to regenerate, treatment 4 would provide extra time, since autotomy of the contralateral leg would provide the necessary delay. The results are given in Figure 26. Both the sham-operated controls and the leg-autotomy-plus-nerve-sectioned animals molt early. These nerve-sectioned animals did not regenerate a limb as would be expected since they did not delay their molting cycle. The 20 hour difference between these two treatment groups may be a bowl effect since all four treatment groups were raised in separate bowls in the incubator. The leg autotomized group shows the typical early non-regenerates, a plateau and then the molting of the regenerates. The nerve-sectioned group with the contralateral leg autotomized shows the typical plateauing effect. Of the 21 animals of this group which delayed their molting cycle to regenerate the contralateral limb, only 4 showed partial regeneration.

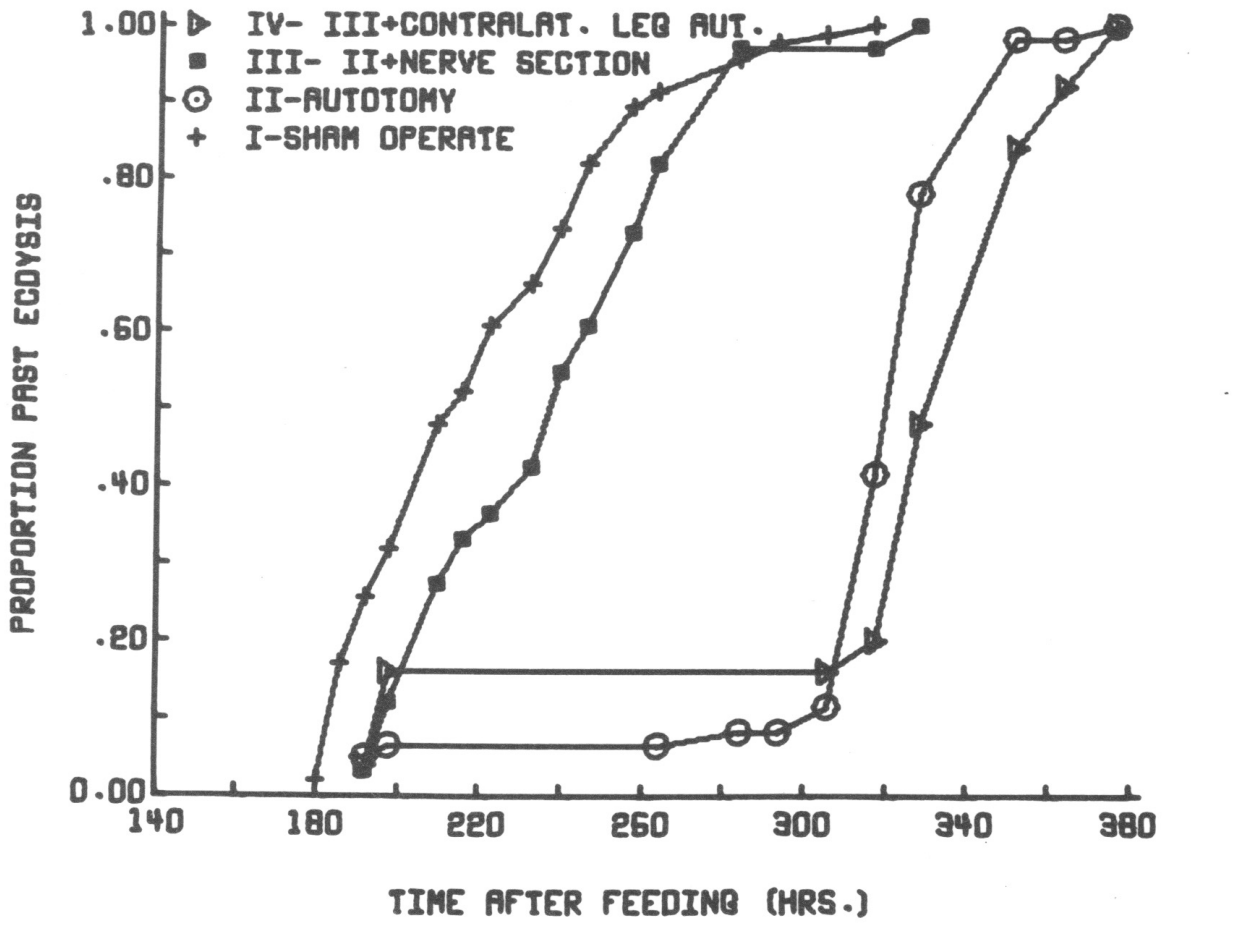


Fig. 26. The effect of time on the regeneration of nerve-sectioned autotomized limbs. Extra time for regeneration was provided nerve-sectioned and autotomized limbs by autotomy of a contralateral limb (group IV). Despite the extra time provided, only 4 group IV animals showed even partial regeneration of non-irritable, non-functional legs.

The failure of nerve-sectioned legs to regenerate is not explained by lack of the usual delay time. The leg stump must be innervated if it is to regenerate. This is consistent with the observation of Bodenstein that in those cases in which regeneration occurred after nerve section, the regenerate was innervated. Even when he removed the ganglion of the autotomized leg, if regeneration occurred, he could find nerves from adjacent segments going to the regenerate.

4. Nerve Section proximal to Leg Ganglion

Another level of interference with the hypothesized nervous feedback on the brain was tested. The ventral nerve cord between the meso- and metathoracic ganglion was chosen as a likely position to intercept the feedback. This would allow the ganglion to maintain its innervation of the leg and therefore the centrifugal effect of nerves on regeneration would not be impaired. One complication

however which arises in ventral nerve cord transected animals is that ecdysis cannot occur due to lack of coordination between the musculature anterior and posterior to the cord section. In order to bypass this difficulty the animals were assayed for regeneration by direct observation through the cuticle of the coxa. Penzlin (1963) has described normal stages of regeneration and approximately when they occur. According to his schedule for seventh instar nymphs, all animals should show clearly visible stages of regeneration six days after autotomy when viewed through the coxal cuticle. I made observations of the coxae of the autotomized legs both at 6 and 8 days to insure that, if regeneration were occurring, I would see it.

A culture of seventh instar *Periplaneta* nymphs was fed and the following four treatments were administered at 84 hours after feeding which should have been before the critical period for regeneration for most of the animals:

T1. autotomy of mesothoracic leg.

T2. autotomy of mesothoracic leg plus section of the meso-metathoracic ganglionic connectives.

T3. autotomy of metathoracic leg .

T4. autotomy of metathoracic leg plus section of the meso-metathoracic ganglionic connectives.

Treatment 2 was included to insure that section of the nerve did not impair the ability to regenerate.

The results are given in Table 17.

Table 17

Treatment	n Non-Regen.	n Regen.	# Dying
1	0	15	0
2	3	9	3
3	0	15	0
4	11	2	2

The controls need mention first. Both the autotomy controls T1 and T3 show a similar result; all of the animals regenerated their limb.

Control 2, which had both autotomy of the mesothoracic leg plus section of the cord showed 3 non-regenerates. This is probably a significant difference from the other controls but the difference is ascribed to complications most likely from puncturing the foregut during the nerve section. The comparison of interest is between the regenerates and non-regenerates of treatments 2 and 4. The proportion of regenerates to non-regenerates of these two treatments are significantly different (Fisher exact test, $p. < 0.005$). The critical factor is the nervous connection between the autotomized leg's ganglion and the, anterior of the animal - - probably the brain.

The data suggest a nervous route of feedback as the mechanism delaying the molting cycle. Pohley and O'Farrell have suggested that the cause of delay is a drain on the humoral concentration of molting hormones caused by the regenerating tissue. These suggestions were based mainly on correlations of size of the regenerate with the amount of delay. Their inference from correlation alone that the amount of tissue causes the amount of delay was not warranted. Experiments directed at the causal relationship of size and delay show that although the amount of delay is correlated with the size of the regenerate, the amount of tissue regenerating does not cause the amount of delay. In the foregoing nerve section experiments, regeneration was shown to rely on nervous connection with the brain and amputation at the autotomy points. These implications lead to the conclusion that molting delay due to leg regeneration is controlled by a nervous feedback mechanism.

IV. DISCUSSION

A. Introduction

The object of this thesis was to characterize the interactions between control mechanisms in development. The model system I chose included the molting cycles of the cockroaches *Blattella germanica* and *Periplaneta americana*. The basic problems were stated to be:

1. The large number of separate goal-seeking units (cells) and levels (tissue, organ, system, organism, social).
2. The large number of interactions between these units and levels .
3. The change in time of the goal-seeking units and levels.
4. The uncertainty of the developmental age of the experimental material.

The first problem dealt with was the uncertainty of developmental age. In section IIIA, I described a simple technique for synchronizing large cultures of cockroach nymphs and adults, utilizing regulation of feeding. This not only aided in a theoretical sense, providing animals of a statistically describable age, but it also made the provision of large number s of experimental animals possible, Section IIIA was devoted to describing some of the parameters which introduce uncertainty into developmental age: temperature, incubator gradients and strain differences. A genetic marker allowed contrasting treatments to be applied to animals which were raised in the same bowl. This made possible finer comparisons without the use of large numbers of bowls within the incubator,

The second problem I dealt with was the description of the change of the system in time. This was intimately related to the uncertainty of developmental age. In section III C-1, I adopted the technique of probit analysis to provide a temporal map of developmental events. This technique, however, gives no clue to the correlation of events within the individual; it merely gives a map of mean times

The basic synchrony of my cultures is probably comparable to that observed in other systems such as molting in *Rhodnius prolixus* (Wigglesworth, 1934) or adult development in the Saturniid silk moths (Williams, 1947) but it is statistically describable, and this is useful in determining sequences of events which cannot be directly observed, such as the critical periods for regeneration, the head and the prothorax. The correlations and causal relations between these events within the individual, which are the interesting development problems, must be sought by pointed experiments.

In order to simplify the task of studying the controls of development I concentrated on a particular goal-seeking system, the locomotory system and its interaction with the molting control system. In particular I studied the relation of leg regeneration to the molting cycle since this had previously been determined to relate in some way to the control of molting (O'Farrell and Stock 1953, Pohley 1965).

The goal of the molting control system is to provide a new and larger cuticle under the old cuticle and subsequently to molt the old cuticle and expand the new one, providing space for the growth of more tissue, a major organismic goal. The goal of the locomotory system is to provide efficient locomotion away from danger and to food, which also serves the general organismic goal of growth.

However , one of the escape mechanisms which the cockroach uses is autotomy of a leg, and leg regeneration with its effect on the molting cycle can conflict with the normally proceeding growth process. If caught by the leg, the cockroach will reflexly lose the leg at one of two autotomy points, the trochanter-femoral joint or the tibia-tarsal joint. Although providing a mode of escape, this process of autotomy alters the efficiency of locomotion and the animal alters its walking pattern, adjusting to the loss of the leg (Wilson, 1966). In addition the animal starts regenerating a leg to replace the lost one. Since the leg is to a great extent an epidermal structure, regeneration understandably conflicts with molting. However, in *Rhodnius prolixus* (Luscher, 1948) the molting cycle is not delayed by regeneration. Molting occurs on schedule even when a limb is amputated and the limb regenerates partially from instar to instar until a complete limb is formed. However this type of partial regeneration is not seen in the cockroach. O'Farrell and Stock (1953) were the first to point out that this all-or-none regeneration in the cockroach was related to a certain critical period after which the regeneration was postponed til the next molt. If a leg was taken off before this critical period, the animal would delay the molting cycle and regenerate a complete leg. The delay of molting was recognized to be peculiar to leg regeneration, since other appendages such as cerci and antennae did not show a molting delay or all-or-none regeneration.

Interaction between locomotory system and molting control system is an interesting case of conflicting goals within an organism. I set out to elucidate the mechanism which resolved the conflict. I will now discuss in more detail the experiments leading to my model of the control of molting and regeneration with comments on the experiments of other workers.

B. Feeding and the Control of Molting in the Cockroach

Feeding is an extrinsic control of the molting cycle which was shown (Section IIIA) to have an initiating effect upon molting. By some pathway feeding must affect the control axis of molting. It is presumed but not proven that this effect of feeding works in a similar way to the control of molting in the blood-sucking bug *Rhodnius* in which feeding distends the abdomen and stimulates stretch receptors which in turn activate the brain (Wigglesworth 1934, Van der Kloot 1960). Abdominal stretch receptors (Finlayson and Lowenstein, 1958) and pharyngeal feeding receptors (Davey 1962) have been described in the cockroach, which could mediate the feeding stimulus I described in section IIIA . Feeding has been shown to control adult female reproductive cycles in a number of species of roaches, including *B. germanica* (Scharrer 1946, Johansson 1955, von Harnack 1958, Roth and Stay 1962, Engelmann 1964, Roth and Barth 1964). This feeding affect on reproduction is not a nutritional effect since cutting the alatal nerves will stimulate yolk deposition in the absence of feeding in *Leucophaea maderae* (Johansson 1955) and also cutting the ventral nerve cord in *Blattella* adult females will cause yolk deposition in a female which is already carrying an egg case (Roth and Stay 1962). Normally as I have shown, an adult *Blattella* female must feed again after parturition before yolk deposition can occur.

These evidences would tend to support the idea that feeding is operating on the neuroendocrine centers in the nymphs and adult female to control the endocrine cycles. Among the paurometabolous insects in which the adult stage resembles the nymphal stages in basic structure and habits, it is

reasonable to think that many of the control mechanisms of the larval stage persist in the adult. This is certainly true in the ametabolous apterygote insects in which molting still occurs after the animals have matured. The molting and reproductive cycles of these insects still maintain a constant relation to one another, the animals are never producing eggs while they are molting and vice versa (Watson, in prep.). It is reasonable therefore that a feeding mechanism might control molting in the nymphal stages and continue similarly in the adult, controlling the female reproductive cycle.

C. Events of the Molting Cycle

Certain events occurring during the stadium were singled out for a study of their relation to the controls of molting and regeneration. Mitosis of the epidermis was observed by Wigglesworth (1940, 1963) to be an early event in the molting cycle of *Rhodnius prolixus*. He demonstrated that the epidermal proliferation triggered by the molting hormone was a response to mutual separation of the cells rather than a direct effect of ecdysone and concluded that the control of cell division was a local phenomenon. My evidence would concur with his notion that epidermal mitosis is associated with molting and not merely a response to feeding. Section III B 3 shows that when the molting cycle is delayed by regeneration, general proliferative mitosis of the epidermis is also delayed. However the differentiative type divisions which produce dermal glands and bristle organules, and occur before the proliferative divisions in the epidermis, are not delayed and are perhaps purely local phenomena.

All described events of the molting cycle subsequent to mitosis in the epidermis will occur in the absence of the brain once the brain critical period is past. However the molting cycle can still be stopped by ligating off the prothorax containing the prothoracic glands. This treatment is presumed to stop the buildup or supply of ecdysone. In any event it is an indication that the molting cycle can be halted even though it has begun, to the extent that the epidermal mitosis has begun. The last point when the molting cycle can be stopped by removing the endocrine glands is close to the time when cell division in the epidermis stops. It is interesting that Sehnal (in prep.) has shown that the sensitive period of the cells to ecdysone stimulation of a new cuticle production in the development of *Galleria* seems to be the G-phase of the cell cycle. This would substantiate the hypothesis that the cells need a continuing supply of ecdysone at least until the last epidermal mitoses have finished and are in the G-phase. It may remain that ecdysone still has an effect on earlier events perhaps at a lower dose level as Buckmann's (1962) evidence in the puss moth, *Cerura vinula* show.

D. Events Prior to the Molting Cycle

If the start of the molting cycle is taken as the beginning of proliferative epidermal mitosis, what type of events occur before the molting cycle begins? Work using colchicine to collect mitoses shows that differentiative types of mitoses appear at the beginning of the molting cycle, presumably for dermal glands and nerve organules. Also some cell death was noticed during this period and this was linked with a possible mechanism allowing every cell to divide when epidermal proliferation begins. However the evidence for division of every cell is weak since it was impossible to collect colchicine mitoses for long enough times due to the disintegration of the colchicine mitoses after 8 hours. Cell division must occur over a relatively short time span in particular regions of the epidermis since with 8

hour colchicine treatments areas of almost total collection of epidermal cells in colchicine mitoses were found.

Another event which occurs before epidermal proliferation is division of cells within the intersegmental muscles. These divisions are presumably of undifferentiated reserve cells since muscle fiber nuclei have never been known to divide. Therefore these divisions would be differentiative divisions resulting in muscle growth.

It is not surprising that regeneration which represents a rather spectacular differentiation must also occur before both the molting cycle and proliferative growth of the epidermis. This would *seem* to be the major conflict between the goals of control of molting and control of regeneration. Previously I had the impression that the reason regeneration could not occur during the molting cycle was because the epidermal cells were producing a new cuticle. Now it seems more likely that the reason regeneration cannot occur after molting begins is that differentiative processes must occur in this early period before proliferative growth of the epidermis starts.

E. The Nature of the Regenerative Process in the Cockroach

A question arises as to what exactly is regeneration in the cockroach? What is happening in the delay provided? The delay of molting due to regeneration of a femur-tibia-tarsus is about 72 hours in fourth instar *Blattella* nymphs and 100 hours in fourth instar *Periplaneta* nymphs. Surprisingly enough, Penzlin (1963) studying the progress of the regenerate in *Periplaneta* reports that mitoses are not seen in the regenerate until the fourth day.

Although he did not use colchicine and therefore may have missed early mitoses, this observation raises serious questions about what is happening during the delay period if it is not a period of mitotic growth of the regenerate. Penzlin (1963) reported that the first 3 days are a time of healing of the wound, accumulation of blood cells, establishment of a blastema and retraction of the epidermis from the trochanter and distal part of the coxa in order to make space for the regenerate.

The fact that growth of the regenerate is not taking place during the delay period can be reconciled with the results of my experiments. I have demonstrated (Section III E 3-5) that in the regeneration of a leg the length of the delay causes the ultimate size of the regenerate at the next molt and not vice versa. Since as discussed above, the major growth of the regenerate does not occur during the delay period, it would be impossible for this growth to be the cause of the delay as postulated by Pohley (1966) and O'Farrell andStock (1959). However it is still possible that some process, perhaps differentiative rather than growth, associated with the leg during the delay period could determine the amount of regenerative growth which occurs when that growth starts.

A candidate for the process associated with the leg which might determine both the delay time and the amount of regeneration is the regeneration of motor innervation of the leg. Jacklet and Cohen (1967) have demonstrated that when nerves innervating leg muscles are cut, a regenerative process includes some histochemically visible RNA accumulation in motor cell bodies of the nerves cut. This morphological activity of the nerve cell bodies is continued until re-innervation, as tested

electrophysiologically, is completed.

A similar process may be occurring during regeneration after the autotomy of a limb. The molting process could be delayed by relaying the information of this ganglionic regeneration process to the brain, to prevent its last roles in initiating a molting cycle. As I have demonstrated (Section III F 4), nervous connection between the ganglion and the anterior is necessary for the molting cycle to be delayed and limb regeneration to occur. The nervous connection necessary for delay to occur is most likely between the ganglion and the brain since mapping experiments (Section III G 1) showed that the critical period for the brain and the critical period for regeneration occur in close connection.

Thus it is reasonable to think that a nervous process occurring during the delay of molting could control the amount of regeneration which results.

Another aspect of regeneration in the roach which I have dealt with briefly (Section III E l) was the ultimate form and size of the regenerate after a number of molts. The regenerate differs in some respects from the non-regenerate leg:

1. The regenerate has a different tracheal trunk supply (Penzlin 1963).
2. The regenerate has 4 tarsal segments as opposed to the non-regenerate's (Brindley 1897).
3. The density of femoral spines is increased in the regenerate compared to the non-regenerate.

These differences in structure could be the result of tissue level interaction in the regenerating leg. Wigglesworth (1954, 1959) has demonstrated that injured epidermal tissue of the blood-sucking bug *Rhodnius prolixus* in its regenerative process, competes for tracheoles. Epidermal cells send out processes which attach to the tracheoles and pull them to the cell to provide the needed oxygen for regeneration and growth. This tissue interaction between the tracheae and epidermal cells may be the cause of a different tracheal supply in the regenerated leg. Bohn (1965) has described segmental gradients in the limbs of the cockroach, *Leucophaea maderae*, similar to the gradients described in the abdominal and leg segments of *Rhodnius* (Locke 1959, 1960, 1965, 1966b). The number of segments of the tarsus and the number of spines on the femur would be integrally related to these gradients. In particular the number of tarsal segments is fixed early in regeneration and presumably the gradients described by Bohn (1966) would be established at that early time. The interesting developmental problem is how these gradients are established in embryonic development and differently in the regenerate. Turring (1952) describes a model by which standing gradients of morphogens can be established in a small structure and may result in the stimulus for such biologically observable patterns as bristle number and pattern in *Drosophila* (Sondhi, 1963). Sondhi's work shows that a pre-pattern where bristles are to develop exists in the head epidermis of *Drosophila*. Various mutants were used to demonstrate that the expression of the pre-pattern was independent of the existence of the pre-pattern. By the Turring model of pattern formation a pre-pattern would be established in the early regenerate, which would result in an expressed pattern of segmentation as regeneration progressed. The question still remains, why does the regenerate have different patterns of structure from the non-regenerate? Reasoning according to Waddington (1966), the regenerating leg had undergone a crisis and in stabilizing had oriented about a new equilibrium or 'tractor surface' as he calls it. This might involve a new prepattern or an expression of an existing but latent pre-pattern which the regeneration uncovers, similar to certain mutants in *Drosophila*.

F. The Nature of the Feedback of Regeneration on the Molting Cycle

The mechanism of regeneration, delaying the molting cycle, may be a nervous feedback mechanism on the neuro-endocrine control of molting. The critical period for regeneration occurs close to if not simultaneously with the brain critical period (Section III C-1). Nerve section between an autotomized leg's ganglion and the brain prevents regeneration (Section III F 4). Demonstration of the dependence of proper molting delay on amputation at the autotomy points, also suggests a nervous mechanism (Section II F 1).

The other main hypothesis concerning the feedback (O'Farrell , Stock, Rae, Morgan 1954, Pohley 1964) states that the regenerating tissue reduces the level of the molting hormone in the blood and thereby delays the molt. Although this may be true for *Ephestia* (Pohley, 1964) it seems unlikely in the cockroach for a number of reasons. O'Farrell and coworkers base their conclusion on several correlations. First, they have observed that mitosis occurs in the brain lobes, the prothoracic glands and *corpora allata* before the proliferative mitoses occur in the epidermis. They use this evidence to support the idea that the brain hormone and molting hormones must already be in the blood at this early time and thus the only reasonable way for the regenerate to prevent molting is to reduce the level of the molting hormone in the blood. However, all the mitoses they have observed may be differentiative type divisions, For example, Gymer and Edwards (1967) have shown that the sixth abdominal ganglion of the house cricket, *Acheta domesticus* has its full complement of nerve cells in the first instar. The only cell divisions that occur from instar to instar are in the glial cells . If this is true in general for the central nervous system of Orthopteroids, then O'Farrell and co-workers may have been observing differentiation of glial cells in the brain and the observed divisions may have had little to do with the actual hormonal levels of brain hormone and ecdysone in the blood. They also suggest that it is the regenerating muscle that causes the drain of molting hormone because the cercus, which cannot delay the molting cycle, has no muscle. This is however only circumstantial evidence and might be balanced by the evidence which argues that the reason the cercus does not delay molting is because it has no autotomy points and therefore no intelligible nervous mechanism for delaying the molt. Moreover, Penzlin (1963) has shown that during the major part of the delay period, no mitoses are seen in the blastema which will form the muscle. It seems unlikely that muscle growth could significantly delay the molting cycle. Sham operations in which muscle damage occurs does not delay the molt to any marked degree. The evidence suggests that the mechanism for delaying molting is a nervous feedback on the brain which prevents the last endocrine role of the brain in initiating the molting cycle.

G. The Role of Nervous Mechanisms in Controlling Development

It is often more efficient to route stimuli from the external and internal milieu through some integration center in order to form a definitive message to be sent to target organs (Scharrer & Scharrer 1963). It is therefore not surprising that the central nervous system plays such an important role in controlling development in insects (Edwards 1966). In the female reproductive cycle of *Blattella* sensory feedback from ootheca in the bursa of the female prevents maturation of additional eggs (Roth and Stay 1962). In some species of cockroaches reproductive behavior is released by feeding while in others feeding is induced by copulation (Roth and Barth 1964). The cockroaches are therefore an interesting group to study control mechanisms of development.

GLOSSARY OF TERMS

ANOVA (Analysis of variance)	A method of decomposing measurements into independent components and testing their significance. The components may be attributable to various effects such as experimental treatment effects or environmental influences. (Cochran & Cox, 1950, p. 53)
confounded	A statistical term used to describe two effects which, by the design of an experiment, cannot be measured independently.
epicuticle	A composite outer-most layer of the insect exoskeleton. (see Locke, 1966)
experiment-wise significance levels	An approach to the problem of performing multiple tests of significance within an experiment. If each test is independent of each other test, a method can be devised which will give a nominal chance of a single test resulting in a false-positive conclusion.
ligation	An experimental technique used to isolate one part of the insect body from another. Head ligation was performed using strands of finely divided dental floss but this technique would not work for ligation between major body segments because the sclerites were too brittle to be compressed by the ligature without splitting and loss of blood. An improved technique involved severing the animal at the ligation point with a razor blade and dipping the open wound in liquid low melting point wax (39C). Animals ligated in this way have a high survival rate.
nymph	The larval stage of an insect which undergoes gradual metamorphosis .
organule	A structure made up of a limited number of specialized cells. A dermal gland in the cockroach is an organule composed of two cells - a gland cell producing a secretion and a duct cell which forms an epicuticle lined duct through the cuticle to carry the secretion of the gland cell to the surface of the cuticle. (after Lawrence, 1967) *
pen inbreeding	A breeding program in which the offspring of one culture of animals are inbred at random as opposed to strict inbreeding in which only siblings are allowed to mate.
physiological age	The age of an animal in terms of its physiological and developmental state, which may not correspond to its age in time.
sclerite	Hardened plate-like portions of the insect exoskeleton.
tergite	A dorsal sclerite.

* This definition of an organule is in error. The basic dermal gland organule is made up of 4 cells (not 2): a gland cell, a duct forming cell, a socket forming cell and a forth cell (perhaps a stem cell for further development).

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CONTROLS OF DEVELOPMENT IN COCKROACHES

"An Abstract"

by

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CONTROLS OF DEVELOPMENT IN COCKROACHES

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ABSTRACT

Availability of food extrinsicly controls both the molting and reproductive cycles of the roaches, *Blattella germanica* and *Periplaneta americana*. The progressive increase in asynchrony of larval development when food is continuously available is prevented by removing food before each ecdysis and replacing it after all animals have finished ecdysis. This method was used to provide experimental animals. Differences in molting cycle length and synchrony were found in three strains of *B. germanica*, Orlando, Chlordane Resistant (Ross and Cochran, 1966, J. Heredity 57, 221-226) and New York. The strain Orlando molts 125 hours after feeding with the highest synchrony (S.D.= 8 hrs at 29ºC). A genetic marker enables raising two treatment groups in the same bowl, a simplification of experimental design. Probit analysis was used to provide a map of the mean times, variability and sequence of events in synchronized colonies of cockroaches. The time when the brain is no longer needed to support a molting cycle is closely associated with the time after which regeneration is postponed until the next molting cycle. This suggests that regeneration delays molting by delaying the last brain event necessary to initiate molting. The time when the prothoracic gland, source of molting hormone, is no longer needed to support molting is close to the end of epidermal cell proliferation and the first events related to production of a new cuticle. Using colchicine to collect dividing cells, I studied the pattern of mitosis in the epidermis. The differentiative divisions of specialized epidermal structures, dermal glands and sensory organules, occur before general epidermal proliferation. Epidermal proliferation starts simultaneously at the posterior of each abdominal tergite and proceeds anteriorly ending at the anterior edge of the intertergite. Epidermal proliferation stops locally before the first structural events in production of a new cuticle begin.

Regeneration delays epidermal proliferation and subsequent events of the molting cycle to the same extent. The existing theory (O'Farrell et al. 1960, Acta Soc. Ent. Csl. 317-324) that the amount of tissue regenerating dictates the amount of delay could not be supported. Animals with strikingly different sized left and right metathoracic legs were produced by prior regeneration. There was no difference in delay associated with autotomy of a small versus a large tarsus even though the amount of delay is a third degree polynomial in length of the regenerate. Also, autotomy of a second tarsus has the same additive effect as autotomy of a second femur, which is not reasonable if delay is caused by the size of the regenerate. I conclude that the amount of delay is correlated with but not caused by the amount of regeneration. Amputation at non-autotomy points does not allow the proper delay of the molting cycle for regeneration to occur. Nerve section between a leg ganglion and the brain prevents the delay of molting needed to regenerate a leg. Nervous feedback from the ganglion of the autotomized leg is suggested as the mechanism by which leg autotomy delays the molting cycle.