Reference: *Biol. Bull.*, 137: 321–331. (October, 1969)

HORMONAL CONTROL OF IMAGINAL DISC REGENERATION IN *GALLERIA MELLONELLA* (LEPIDOPTERA)

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A variety of experiments have demonstrated that regeneration delays molting in insects. A penetrating study of this matter was undertaken by O'Farrell and Stock (1953) who investigated the regeneration of metathoracic leg and its effect on molting in larvae of the cockroach, *Blattella germanica*. They removed the limb at various times in the insect's molt cycle and showed that regeneration retarded molting. When a limb was amputated early in an instar, the insect regenerated the limb before molting and the instar was lengthened. When a limb was amputated after the prothoracic glands had begun to secrete the molting hormone, the insect molted on schedule without regeneration, and regeneration occurred at the next molt (O'Farrell and Stock, 1953, 1954, 1958; Stock and O'Farrell, 1954; O'Farrell, Stock and Morgan, 1956; and O'Farrell, Stock, Rae and Morgan, 1960). These results were extended by Pohley who examined the delay in molting caused by amputating the antennae of larvae of *Periplaneta americana* (Pohley, 1959) and by extirpating the imaginal wing discs of larvae of the flour moth, *Ephestia kuhniella* (Pohley, 1960). He suggested that the delay in molting was due to the regenerating tissues either inhibiting the growth and differentiation of other tissues or reducing the concentration of molting hormone in the insect.

In addition to these studies of the effects of regeneration on the control of molting, the possible role of the molting hormone in controlling regeneration has also been examined. For example Bodenstein (1955) has analyzed the effects of parabiosis and prothoracic gland transplantation on the regeneration of limbs in adult cockroaches. However, these and other studies focused primarily on the regeneration of cuticular structures which become evident only at a molt and perforce depend on the molting hormone. The present experiments examined the role of the molting hormone in the regeneration of imaginal discs, independently of whether a molt occurred. Our experimental objects were the imaginal wing discs of the larvae of the wax moth, *Galleria mellonella*. We examined regeneration in larvae with actively secreting prothoracic glands, in larvae whose prothoracic glands were removed by ligation, and in larvae without prothoracic glands which received a synthetic molting hormone, ecdysone. The experiments answer the question of whether ecdysone is needed for imaginal disc regeneration and also provide some new information on the mechanism whereby regeneration delays molting.

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Materials and Methods

Last instar larvae of *Galleria* from a synchronously molting culture were used for the experiments. The larvae were reared according to the procedure of Sehnal (1966). On the day of ecdysis into the last larval instar, they were collected and kept in petri dishes with food at 30° C and 70% R.H. Such animals pupate 8 or 9 days later, within a day of one another.

Crystalline synthetic α ecdysone was generously provided by Messrs. Hoffman La Roche, Inc. and was dissolved in 10% ethanol to a concentration of 750 μg/ml.

The following surgical operations were performed on 4-day old last instar larvae: (a) Extirpation of one or both imaginal forewing discs or extirpation of all wing discs. (b) Extirpation of right forewing disc and cautery of the wound with a hot needle; this produced a circular burn 2–3 mm in diameter. (c) Sham operated controls; the treatment was like (a) excepting the discs were not extirpated. (d) Ligation of larvae with nylon thread between pro- and mesothorax (to remove the brain and the prothoracic glands). Until six days after the last larval molt this kind of ligation produces "dauer" larvae which survive and respond to tactile stimuli for more than 40 days after ligation. (e) The same ligation as (d) but accompanied by extirpation of the right hind wing disc at specific intervals after ligation. (f) The same operation as (e) but accompanied by injection into each larva of 0.75 μg of synthetic α ecdysone 14 days after ligation. Each group consisted of 20 larvae. A total of 400 larvae were used.

The technique of extirpation of the wings was similar to that developed by Kroeger (*cf.* Schneiderman, 1967). The larvae were etherized for 3 to 4 minutes and placed in a wax-lined dissecting dish filled with insect Ringer solution. On either side of the dorsal midline of the meso- and meta-thoracic segments of the larvae, there is an opaque white spot which marks the position of the tracheae entering the wing disc. A small longitudinal incision was made in this region with a pair of scissors and the imaginal disc was carefully pulled out and then cut off. The cut ends of the skin were lightly pressed together with forceps and the wound sealed with melted paraffin. The operated larvae were then dried on filter paper and transferred to a petri dish with food and kept at 30° C.

The regeneration of the wing discs was examined in the normal larvae at selected intervals, in the ligated groups 30 days after extirpation and in the ecdysone-injected groups 6 days after injection. Simultaneously, the condition of the wing discs on the unoperated side was also recorded.

Results

Normal development of the wing discs

In *Galleria* kept at 30° C, the final larval stadium and prepupal period lasts 8 days for males and 9 days for females. The imaginal wing discs in the 4-day old larva are small flattened epithelial pouches, one on each side of the meso- and meta-thorax, just internal to the epidermis. Each wing analagen contains the epithelial disc tissue which is invaginated into and is covered by a thin layer of epithelium, the peripodial sac, and is supplied with a cluster of tracheae at its base. As the larva grows, the wing anlagen progressively increases in size and

develops conspicuous lacunae. In the prepupa (2 to 3 days before pupation) the wing discs undergo a further increase in size and small tracheae migrate into the lacunae. About 12 hours before pupation, the developing wings are everted to the outside of the body to lie beneath the loose larval cuticle that envelops the prepupa. The pupal wings then expand to pupal size. After pupation they tan within a few hours.

Regeneration of extirpated wings in the unligated larva

The process of regeneration of the wing disc was examined at selected intervals in 4-day old larvae in which the right forewing disc was removed. Regeneration of the extirpated wing disc was not evident until 5 days after the operation. However, on the fifth day, a small regenerated wing disc was evident on the extirpated side. It was thin, transparent and about $\frac{1}{8}$ the size of the disc in the unoperated side and had an accumulation of tracheae at its base. It is interesting to note that the unoperated discs and the other tissues of the larva failed to grow or differentiate further and remained in the same stage of development they were when the disc was extirpated. By the seventh day after the operation, the regenerated wing disc had further increased in size and was nearly $\frac{3}{4}$ the size of that on the unoperated side. The unoperated disc also resumed their development. In both the regenerated and control wing disc, lacunae appeared and tracheae began to migrate into them. By 8–9 days after the operation, the regenerated and control wing discs had grown nearly to the same size, differentiated further, and resembled the wing discs of normal larvae immediately before pupation. When the operated larvae pupated, the pupal wings on both sides looked similar and the resulting adults developed normal wings. Commonly, the regenerated wing was about 15% shorter than the wing on the unoperated side.

TABLE I

Results of various surgical operations on 4-day old last instar larvae of Galleria mellonella

Group	Operation	Per cent regenerating	Days to pupate of those regenerating (± = s.d.)	Days to pupate of those not regenerating (± = s.d.)
I	Untreated larvae	—	—	4.5 ± 0.5
II	"Sham" operated controls	—	—	5.6 ± 0.48
III	One forewing disc removed	85	9.6 ± 0.59	6 ± 0.66
IV	Both forewing discs removed	100	13.9 ± 1.2	—
V	All four wing discs removed	100	18.5 ± 1.5	—
VI	Extirpation of one forewing disc plus cautery of the wound	—	—	5.7 ± 0.46

Each group contained 20 larvae.

Effects on pupation of extirpating one, two or four wing discs

The results of extirpating either 1, 2 or 4 wing discs from 4-day old larvae and its effect on their pupation is shown in Table I. The controls for the wing

disc extirpation experiments were "sham" operated larvae. All larvae survived the "sham" operation or removal of 1 or 2 of their wing discs. However, when all 4 wing discs were removed 30% of the larvae died as a result of the surgery. The table records the time it took each group of larvae to pupate. Each group consisted of approximately equal numbers of males and females. In almost every group the females pupated on the average a day later than the males; however, since this fact is irrelevant to the present experiments the results for both sexes have been pooled.

Table I shows that the wound caused by the "sham" operation (group II) delayed pupation by one day.

After extirpation of one wing disc (group III), 17 of the 20 larvae regenerated the missing wing disc. Pupation was delayed in these larvae by an average of 5 days and occurred 8–10 days after the operation. The remaining 3 operated larvae failed to regenerate and contained only a small healed stump measuring $\frac{1}{10}$ the size of normal wing disc. These larvae pupated 5, 6 and 7 days after the operation, an average delay of 2 days.

TABLE II

The results of extirpating right hindwing imaginal discs from ligated last instar larvae of G. mellonella

Days after ligation	Per cent of larvae with regenerated wing
0	75
2	75
4	65
6	25
8	15
10	10
14	10

Each group contained 20 larvae.

After removal of both forewing discs (group IV) all of the larvae produced a regenerate and pupated. Pupation of these larvae was delayed by 9 days.

When all wing discs were removed (group V), all of the operated larvae that survived produced a regenerate. Pupation of these larvae was thus delayed by 14 days.

Effect of cautery of the wound on pupation

When the right forewing discs were extirpated from larvae and the overlying epidermal region of the wound was cauterized (group VI, Table I), the larvae molted into pupae 5–6 days after the operation. There was thus only 1 day delay in pupation. The resulting pupae had no right forewings.

Regeneration of wing discs in ligated larva

Four days after the last larval molt larvae were ligated between pro- and meso-thorax to produce "dauer" larvae. Dissection of these larvae at various

times after ligation revealed that the further growth of the wing discs and other internal organs had stopped. The results of extirpating wing discs on different days after ligation are recorded in Table II. Each experimental group contained 20 ligated larvae. Larvae were examined for regeneration 30 days after extirpation. In all cases ligation stopped further growth of the unoperated left hand wing disc and other internal organs. However, as the table reveals, when the right hind wing discs were extirpated 0, 2 or 4 days after ligation, more than 65% of the larvae regenerated their right hind wing discs. The remaining 35% failed to regenerate. In the successful cases, dissections showed that the regenerated wing discs had the same shape as the normal regenerated wing discs produced by unligated larvae, but they were thinner than normal regenerates and quite transparent. They were nearly $\frac{3}{4}$ the size of their counterparts in the unoperated side. In most cases, there was accumulation of tracheae in the base of the regenerated disc similar to that seen in normal discs.

When discs were extirpated 6–14 days after ligation, the percentage of larvae in which regeneration occurred decreased markedly from 65% to 10%. The mor-

TABLE III

Effects of various treatments on last instar larvae of G. mellonella *ligated for 14 days*

	Per cent regenerating	Per cent molting	Per cent pupating	Per cent remaining larval
Right hind wing disc extirpated on 14th day	10	0	0	100
Right hind wing disc extirpated on 14th day and 0.75 μg of ecdysone injected within 15 min.	65	100	35	0
Right hind wing disc extirpated on 14th day and 0.75 μg of ecdysone injected after 24 hours	0	75	75	25

Each group contained 20 larvae.

phology and size of the regenerated wing disc in the 6, 8 and 10 days groups resembled those seen in the 0–4 day groups. However, the few regenerates observed in 14 day group were markedly different. The regenerates were thin and only $\frac{1}{10}$ the size of the wing disc on the unoperated side. There was no further growth in the wing discs on the unoperated side which remained at the same stage of growth as on the day of ligation.

Effect of ecdysone on the regeneration of wing discs in ligated larvae

From the experiments reported in section 5 it is clear that ligated larvae gradually lose the ability to regenerate. Is loss of regenerating capacity in these ligated larvae associated with the loss of ecdysone, a decay in the effects of ecdysone, or the absence of some other factor? To answer this, the experiments summarized in Table III were conducted.

Groups of 20 larvae were ligated between pro- and meso-thorax and their right hind wing discs were extirpated 14 days after ligation. One group received

no further treatment, (the same group described in the last line of Table II). Only 10% of these larvae regenerated, and these produced a feeble regenerate. A second group of 20 larvae was injected with 0.75 μg of crystalline ecdysone *within 15 minutes after extirpation of the disc* (*i.e.* approximately 10 μg/gm live weight). Within 6 days, 65% of such treated larvae had regenerated their wings. The regenerated wing discs were well-formed and resemble those produced by larvae in which wings were extirpated on the day of ligation. It consisted of disc tissue with a peripodial sac and with tracheae in its base. It was about half the size of the wing disc on the unoperated side. It is also noteworthy that, as a result of ecdysone injection, the wing disc on the unoperated side increased in size and the tracheae began to migrate into the lacunae of the disc tissue. However, development stopped at this point. The ecdysone injection promoted the regeneration of the extirpated disc and also the further development of the unoperated wing disc. The ecdysone also initiated the process of molting. In most cases the old cuticle of the larva could be separated from an underlying new cuticle. In a few cases apolysis occurred, but it was difficult to determine whether or not the underlying epidermis had secreted a new cuticle.

The 35% of ligated larvae which failed to regenerate their wing discs after injection of ecdysone went on to pupate within 4 days of the injection. The wings on the unoperated side grew, differentiated and everted in a normal manner.

A third group of 20 larvae received the injection of 0.75 μg of ecdysone *one day after wing disc extirpation* instead of 15 minutes after extirpation. Different results were obtained. As Table III reveals, the stump healed and no regeneration occurred. Moreover, most of the larvae made some attempt to pupate 3 days after the injection and produced patches of recognizable pupal cuticle. In these larvae the unoperated wing discs grew and differentiated to a stage similar to that seen in normal early prepupa.

Discussion

The necessity of ecdysone for regeneration of imaginal discs of Galleria

The present experiments bear on two questions, namely: the necessity of ecdysone for imaginal disc regeneration and the mechanism whereby regeneration blocks development and molting. A clear answer was obtained to the first question; the second question remains unanswered.

The role of ecdysone in imaginal disc regeneration in *Galleria* was demonstrated in experiments where wing discs were extirpated from larvae whose brains and prothoracic glands had been removed by ligation. When the discs were extirpated from these larvae 0 to 14 days after ligation, the percentage of larvae in which regeneration occurred decreased markedly from 75% to 10%. This defect in ligated larvae was repaired by injecting crystalline α-ecdysone which enabled the larvae to regenerate their extirpated wing discs. These findings lead to the conclusion that regeneration of imaginal discs requires ecdysone. This conclusion explains not only the present results on *Galleria,* but also the results of prothoracic gland transplantation and parabiosis experiments on regenerating adult cockroaches performed by Bodenstein (1955) and are consistent with his prediction that "the loss of regeneration . . . is caused by the absence of thoracic gland hormone" (Bodenstein, 1959, page 3).

The effects of ecdysone on the regeneration of imaginal discs finds some parallel in recent experiments of Oberlander and Fulco (1967) who showed that the increase in size of wing discs of early prepupae of *Galleria* maintained *in vitro* ceased in the absence of α ecdysone and continued in its presence. Further evidence for an effect of ecdysone on imaginal disc regeneration comes from a recent report on the development of imaginal discs in *Drosophila* (Postlethwait and Schneiderman, 1968). When intact imaginal discs of mature *Drosophila* larvae were implanted into adult abdomens, the increase in size of the discs was significantly promoted by injecting an ecdysone. However, neither of these experiments nor the present experiments enable us to decide whether the normal growth of imaginal discs during larval life requires ecdysone.

The ability of ligated larvae to regenerate wing discs up to 10 days after ligation may be due to residual effects of ecdysone that persisted in the larva after ligation. Williams (1968) has suggested such residual or "covert" effects of ecdysone decay over the course of several days. The present experiments reveal that after 14 days the residual effects of ecdysone in ligated *Galleria* larvae either completely disappear or were so slight that the extirpated wing discs failed to regenerate. The persistence of these residual effects of ecdysone may also explain both the observation of Pohley (1961) that mature larvae and early prepupae of *Ephestia* deprived of both brain and prothoracic glands still regenerated wing discs that were removed at the same time the glands were extirpated and also the interpretation that imaginal disc regeneration in *Ephestia* seems to be independent of the molting hormone (*cf.* Wigglesworth, 1965, page 101).

Although ecdysone seemed to be necessary for regeneration in *Galleria*, it was not always effective. In the present experiments 35% of larvae which received 0.75 μg of ecdysone 14 days after ligation failed to regenerate a wing and went on to pupate (Table III). It is significant that this group pupated two days before the 65% that regenerated. To understand this result it is necessary to appreciate the effects of different concentrations of ecdysone on the epidermis and other chitogenous epithelia. Low concentration of ecdysone stimulates growth and cell division whereas high concentration of ecdysone stimulates cuticle secretion and may actually block DNA synthesis, cell division and growth (Krishnakumaran *et al.*, 1967, page 36; Madhavan and Schneiderman, unpublished; Postlethwait and Schneiderman, 1968). In present experiments the 35% of the animals that pupated instead of regenerating a disc after an injection of ecdysone may have been more sensitive to ecdysone and therefore started to secrete a cuticle which, perforce, prevented any regeneration. This interpretation is strengthened by recent observations that when larger doses of ecdysone (10 μg/animal) were injected into ligated *Galleria* larvae with one wing disc extirpated, all of the animals pupated (20 of 20) without regeneration (Madhavan and Schneiderman, unpublished). Apparently ecdysone promotes regeneration only within a certain range of concentrations. Below this range it is ineffective; above this range it promotes prompt cuticle secretion and blocks regeneration.

Also, it is interesting to observe the behavior of the ligated larvae when supplied with 0.75 μg of ecdysone 24 hours *after* extirpation of the wings. In such larvae the dose of ecdysone injected was inadequate to cause pupation: 75% of the larvae made some attempts to pupate, but secreted only occasional patches of pupal cuticle;

the remaining 25% remained larval. Furthermore, no regeneration occurred. Apparently, when the wound healed in the absence of ecdysone, then the healed stump showed a decreased sensitivity to ecdysone when compared with a fresh wound. The enhanced sensitivity of injured tissue to hormones has long been known in the case of juvenile hormones (*cf., e.g.* Schneiderman and Gilbert, 1959) and it may also be true of ecdysone.

Direct and indirect effects of ecdysone on regeneration

From the results discussed above it is evident that the regeneration of imaginal wing discs is promoted by ecdysone. However, the question remains whether this is due to a direct effect of ecdysone on the stump of the disc itself or to an indirect effect of ecdysone on other tissues, or both? Evidence that ecdysone acts directly on the regenerating stump itself comes from recent experiments of Oberlander (1969) who showed that ecdysone promoted DNA synthesis, tracheal migration and disc elongation in wing discs of *Galleria* cultured *in vitro*. This important result argues strongly in favor of a direct action of ecdysone on the discs. It also appears likely that in addition to any effect it has on the disc tissue itself, ecdysone promotes the development of the tracheal system which appears to be necessary for regeneration.

Thus ecdysone may promote regeneration of imaginal disc directly by acting on the imaginal disc cells themselves and indirectly by promoting the growth of the tracheal system.

The role of regenerating tissue in delaying development and molting

One of the most striking features of wing disc regeneration is that it delays both the development of certain other tissues, such as normal wing discs, and the pupal molt. The delay in pupation is not due to the effect of wounding because larvae pupate on time even when they receive several wounds. The delay in pupation is caused by the regenerating wing disc. Such an influence of the regenerating tissue on the molt cycle has been recorded for *Blattella* (O'Farrell, and Stock, 1953), *Periplaneta* (Pohley, 1959) and *Ephestia* (Pohley, 1960, 1965, 1967).

The importance of the regenerating tissue is clearly seen in the experiments reported here in which the regenerating epithelium was destroyed by cautery. Such larvae pupated without delay and without regeneration. Also, in *Galleria*, the delay in pupation increased when several discs were removed and appeared to be roughly proportional to the amount of regenerating tissue: removal of one disc delayed molting by 5 days, removal of two discs delayed it by 9 days, and removal of four discs delayed it by 14 days. Prolonged delay in molting after extirpations of several structures has also been observed in *Blattella* (Stock and O'Farrell, 1954) and in *Ephestia* (Pohley, 1965, 1967). Similar delays in molting and blocks to development can be produced not only by extirpating imaginal discs, but also by implanting an extra disc into the body cavity of a larva (Kroeger, 1958; Muth, 1961). The implant develops a mirror image of itself and, during this process of "doubling," both further growth of host larval structures and pupation are delayed.

This block to both development and molting can be repaired by ecdysone. In the present experiments when a larva with one wing disc extirpated was injected with ecdysone, both the operated disc and the unoperated disc grew and developed simultaneously, whereas in unligated larvae the unoperated disc "waited" until the regenerated disc had "caught up."

Since regeneration, the larval-pupal development of normal wing discs and molting all require ecdysone, one explanation for the blockage of molting is that the regenerate decreases the effective concentration of ecdysone. It might accomplish this (1) by using up the ecdysone present in the larva, (2) by promoting the breakdown of ecdysone, (3) by suppressing but not totally blocking the secretion of the prothoracic glands either directly or indirectly via the brain. An alternative explanation is that the regenerate (4) by nervous or (5) by humoral means changes the responsiveness of other tissues to ecdysone and (6) perhaps promotes the decay of residual or covert effects of ecdysone. For the present, it is not possible to decide between these explanations.

To conclude, the present experiments demonstrate that ecdysone is necessary for the regeneration of imaginal discs in *Galleria;* however, they fail to discern how the regenerating tissue prevents the growth of normal tissues.

This research was performed during the tenure by one of us (K. M.) of an N. I. H. post-doctoral traineeship in Developmental Biology and was supported in part by a research grant, HE 6320, from the U. S. P. H. S. We wish to thank Dr. A. Krishnakumaran and Mr. John Postlethwait for their helpful comments on the typescript.

Summary

1. These experiments examined the mechanism whereby regeneration delays development and molting in the waxmoth, *Galleria mellonella,* and also the possible role of the molting hormone in controlling regeneration.

2. Removal of one or more imaginal wing discs from four-day old last instar larvae delayed the further development of unoperated discs and the pupal molt, confirming prior observations on *Ephestia, Blattella* and *Periplaneta* by other workers. The delay in molting was proportional to the amount of regenerating tissue: extirpation of one wing disc caused a five-day delay, whereas extirpation of all four discs caused a 14-day delay. When the regenerating epithelium was destroyed by cautery, no delay in molting occurred. Several possible mechanisms for this delay were examined.

3. In another series of experiments four-day old last instar larvae were ligated in such a way as to eliminate both brain and prothoracic glands. When imaginal wing discs were extirpated from these ligated larvae 0 to 14 days after ligation, the percentage of larvae in which the imaginal discs regenerated decreased from 75% to 10%. This defect was repaired by injecting 10 μg/gm of a synthetic molting hormone, alpha ecdysone, immediately after extirpating the wing disc. This treatment enabled the larvae to regenerate the missing disc.

4. It is concluded that regeneration of imaginal discs in *Galleria* requires ecdysone.

5. Additional experiments indicated that ecdysone promoted regeneration only within a certain range of concentrations: a concentration of 10 μg/gm promoted regeneration whereas 100 μg/gm promoted the prompt secretion of cuticle and blocked regeneration.

6. Ecdysone was less effective in promoting regeneration if the wound healed before it was applied. Thus injecting 10 μg/gm *immediately* after wing disc extirpation promoted regeneration, whereas an identical injection *24 hours after* extirpation failed to promote regeneration. This suggests that injured tissues may be more sensitive to ecdysone.

7. Arguments were advanced that ecdysone promoted regeneration of *Galleria* wing discs directly by acting on the imaginal discs themselves and perhaps indirectly by promoting the growth of the tracheal system.

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