Differentiation of Pulsating Regions in Genital Imaginal Discs after Culture *in Vivo* (*Drosophila melanogaster*) [1]

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ABSTRACT After male genital discs of *Drosophila melanogaster* had been cultured for 8 to 19 days in the abdomens of adult female flies a number of them contained rhythmically pulsating regions. The percentage of discs which pulsated was dependent on the age of the donor larvae, the length of culture time, the presence or absence of ring glands and on the age of the host. The best conditions for the differentiation of pulsating discs were an old disc in a young host to which a ring gland from a mature larva was added.

In normal development the genital disc after metamorphosis gives rise to the genital apparatus with morphologically differentiated ejaculatory duct, sperm pump and paragonia (soft parts) which contract rhythmically. In the cultured discs, however, the contractions are not accompanied by morphogenesis.

The prospective fate of the pulsating region of a disc is mostly contracting soft parts of the genital apparatus, whereas the non-contracting area forms mostly chitinous parts (anal plates, claspers, lateral plates). Other discs, e.g. leg discs and wing discs, which in normal development do not form involuntary muscles also do not contract after culture *in vivo*.

It is concluded that the formation of pulsating regions represents a phase in the normal differention of genital discs. Some of the conditions which lead to pulsation are established early in the third instar within the cells which form the pulsating regions. Their full differentiation is influenced by hormones secreted by the ring gland.

Genital imaginal discs of *Drosophila melanogaster* are easily cultured for long periods in the abdomen of adult flies. Even in cultures which have been maintained for several years the cells remain embryonic. They divide, but do not differentiate. They retain their full developmental capacities, however. These can be tested at any time by transplanting the blastema into a larval host. Here they undergo metamorphosis and differentiate into adult structures (Hadorn, '63, '65, '66). In contrast to the genital disc, the eye disc differentiates after only two weeks in an adult milieu (Bodenstein, '43), producing ommochrome pigments (Schläpfer, '63).

During an investigation of proliferation in cultured male genital disc fragments, we discovered a number of implants which contained groups of rhythmically pulsating cells. In this paper we describe this differentiation and consider the following two questions: (1) Under what conditions do these pulsations occur? (2) What is the origin and prospective fate of the contracting region?

MATERIALS AND METHODS

1. *Drosophila stocks*

The wild stock "Sevelen" of *Drosophila melanogaster* was used in all experiments as larval donor and larval host. The adult hosts were fertilized females of a homozygous white-stock (w1-1.5). The animals were reared on standard food (maize, sugar, agar, yeast) at 25° C. The age of the donors and hosts in each experimental series is given in hours after egg laying for the larvae, and in days (d) after emergence for the flies.

2. *Technique*

The imaginal discs were dissected in Drosophila Ringer's solution and cut with

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a tungsten needle. The fragments were then injected into the body cavity of flies or larvae (Ephrussi and Beadle, '36). The discs remained in the adult abdomen for varying lengths of time. They were then removed and checked for the presence or absence of rhythmic contractions under a microscope (magnification: 50 ×). The results are based on an examination of over 300 implants.

3. *Permanent preparations*

Whole mounts of discs and metamorphosed genital structures were fixed for 15 minutes in ethyl alcohol-acetic acid (3:1), and stained with Gomori's hematoxylin (Melander and Wingstrand, '53).

RESULTS

1. *Occurrence and nature of pulsation*

Types of imaginal discs which can pulsate. Male genital discs were removed from mature larvae (114–120 hours old), and cut in the median plane. The two halves were then transplanted individually into the abdomens of one day old female adults. After a culture period of two weeks 16 out of 22 recovered implants pulsated rhythmically in Ringer's (table 1a). In contrast similar experiments with other types of discs gave the following results: Only two out of 26 female genital disc fragments pulsated, while none out of 18 first leg disc fragments and 16 wing disc fragments did so.

Location of pulsating implants. The location of the transplanted male genital disc within the host's abdomen had no relation to the occurrence of the contracting region. Pulsating discs were found at the site of the wound, attached to the gut or the ovary, or floating free in the abdomen.

Extent of pulsating area. Usually, only one or two small regions in an implant contracted. The pulsating area did not appear in the same position in every implant; it could be located at the surface or within the disc. It should be noted, however, that after two weeks of growth in an adult host the morphology of the implanted disc fragment was often distorted.

Nature of pulsations. The amplitude of individual contractions varied greatly, but was not measured. The rate of pulsation ranged from 12 to 60 beats per minute, which is about the same range found for metamorphosed genital soft parts *in situ*. The rate of pulsation was independent of the amplitude of contraction. Thus, a disc with weak contractions may pulsate quickly, while a disc with strong contractions may pulsate slowly. In some discs pulsations continued in Ringer's for three hours after removal from the adult host.

2. *Parameters affecting the occurrence of pulsations in male genital discs*

Culture time. To determine if the time of *in vivo* culture affected the percentage of pulsating discs, mature male genital discs (114–120 hours old) were cut in half and implanted into young female adults (one-day-old). Samples of discs were removed at varying times after transplantation, and the occurrence of pulsations was noted. The results, shown in table 1a, demonstrate that three and five days were insufficient periods of culture for the appearance of pulsations, and that

TABLE 1

The effect of culture time on the occurrence of pulsating regions in male genital discs

Series	Age of discs	Time cultured in adult fly	n	Per cent pulsating discs
	hours	*days*		
a	114–120	3	10	0.0
	114–120	5	10	0.0
	114–120	8	9	11.1
	114–120	11	16	37.5
	114–120	14	22	73.0
b	108–110	15	20	20.0
	108–110	19	10	60.0

n = number of implanted discs.

the best period observed was 14 days. When the source of the discs were younger larvae (108–110 hours old), a culture period of 19 days resulted in a threefold increase of the percentage of implants which pulsated after 15 days (table 1b).

To test the effect of culture time further, as well as to examine the permanence of the state (presence or absence of pulsations) found after two weeks, pulsating and non-pulsating discs were cut in half and reimplanted into adult hosts for a second two week period. At the end of this time 60% ($n = 10$) of the implants from pulsating discs (originally from larvae 114–120 hours old) still contracted. All of the implants from discs ($n = 9$) (orginally from larvae 99–105 hours old) which failed to contract after 14 days also failed to do so after 28 days.

On the basis of the above results all succeeding experiments were performed using a culture time of two weeks.

Age of the donor larvae. The influence of the age of the donor larvae on the occurrence of pulsations in the transplanted discs was investigated using male larvae from three age groups: 99–105 hours, 108–110 hours, and 114–120 hours. In the oldest sample much of the donor population had already pupated, but only the larvae were used as donors. The genital discs were removed, medially fragmented and transplanted into one-day-old female adults as before. The results obtained after two weeks of culture showed that the discs from the oldest larvae produced the highest percentage of pulsating implants, while discs from the youngest donors produced the lowest percentage (see fig. 1, columns a).

Age of the adult hosts. To test the influence of old hosts on the formation of pulsating implants, male genital discs were removed from larvae of three different ages (as above), cut in half, and transplanted into 6 to 7-day-old female adults. The occurrence of pulsations was noted after two weeks and compared with the results obtained from one-day-old hosts, (compare columns a with c for each age group in fig. 1).

While a similar percentage of pulsating discs was formed in both young and old hosts by the 108–110 hours old group, the percentage for younger discs (99–105 hours old) was reduced to zero in the old host, and the percentage for mature discs in old hosts was half that observed in young hosts.

Implanted ring glands. Since the ring gland secretes the hormones which control adult differentiation during normal development, we examined the effect of ring glands on the production of pulsating regions in cultured imaginal discs. Male genital discs from the same three age groups, as above, were medially fragmented and injected into one-day-old female flies, into which two ring glands from mature larva (114–120 hours) had been injected on the previous day. After two weeks in culture the percentage of pulsating discs found was more than double that observed in young hosts without ring glands for both the 99–105 hours and 108–110 hours old groups, and was increased from 80 to 90% for the 114–120 hours old group (compare columns a with b for each age group in fig. 1).

Furthermore, discs from 114–120 hours old larval donors implanted into old hosts with ring glands produced pulsating regions in 65% of the implants as opposed to 35% for discs of the same age in old hosts without implanted ring glands (compare column c with d for the oldest age group in fig. 1).

Proliferation. Since all of the above experiments were conducted with discs which were cut in half before transplantation it was necessary to examine the role of proliferation, if any, in the production of pulsating regions. We observed that male genital discs which were cut in half grew to varying degrees in the adult environment. Pulsations were found in discs that had grown extensively as well as in discs which had grown very little. To examine this question further, whole male genital discs were cultured for two weeks in adult hosts. Although even whole discs grow in an adult milieu, the growth is small compared to that of disc fragments. Fifteen out of sixteen whole discs tested in this manner pulsated — an even higher percentage (93.8% vs. 80%) than for discs of the same age which were medially sectioned before transplantation. In addition there were two or three pulsating areas as

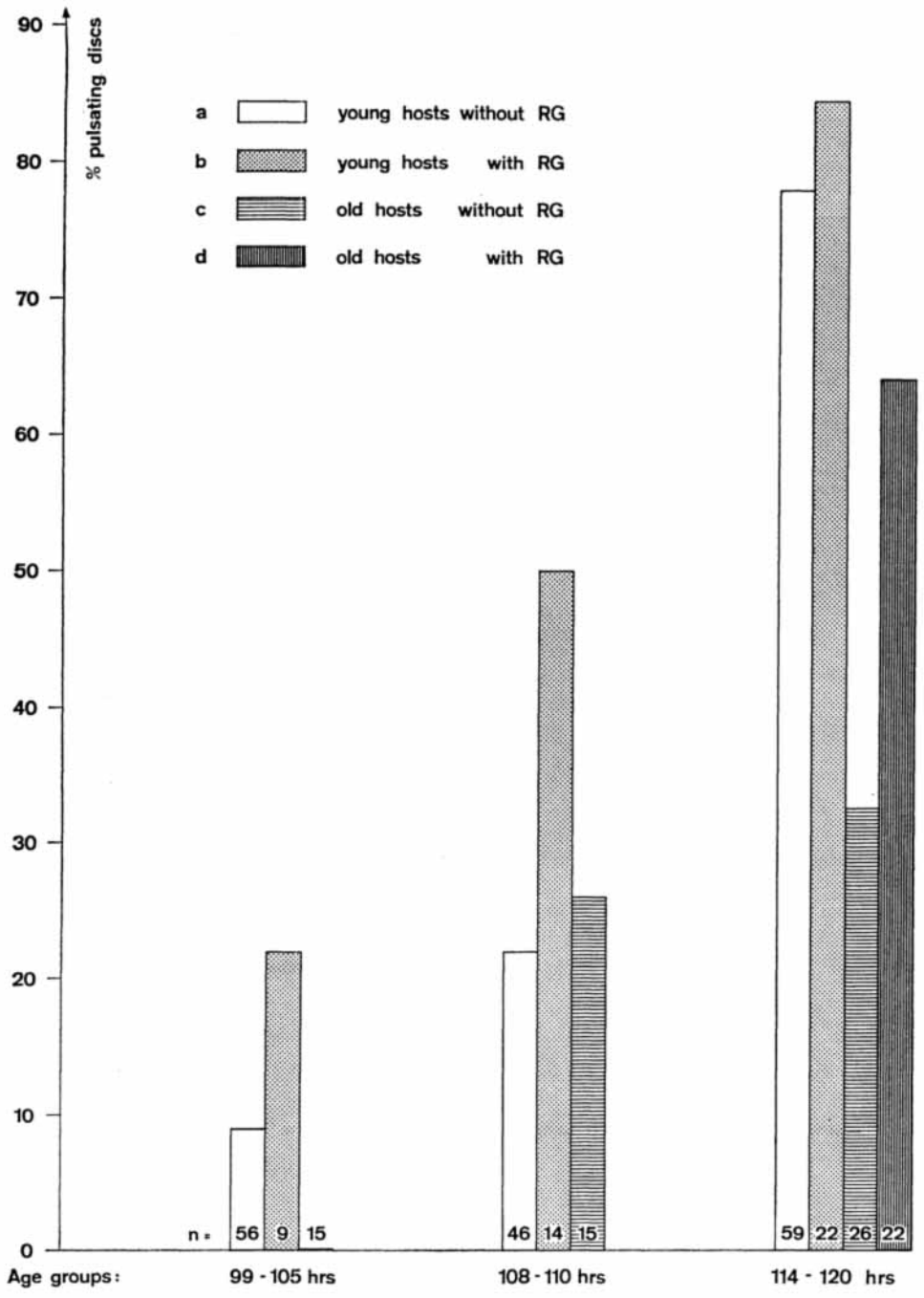

Fig. 1 Effects of age of disc, age of host and presence of ring glands on the occurrence of pulsations in male genital discs. Age of discs is given in hours at time of transplantation. "n" equals the number of discs in each experiment.

opposed to the one or two normally found in implanted half-discs.

3. *Origin and prospective fate of the pulsating region*

Prospective fate of the contractile region. The prospective fate of contracting and non-contracting portions of cultured male genital discs was compared (table 2, fig. 2). Pulsating and non-pulsating regions of the same or other cultured discs were cut out separately with a tungsten needle, and implanted individually into larvae (78–80 hours old). The fate of each implant was then followed after it metamorphosed with the host. An examination of whole mounts of the differentiated structures showed that pulsating regions developed mostly into soft parts, such as sperm pump and ductus, which contract after differentiation, while non-pulsating regions made either mostly chitinous structures (anal plates, claspers etc.) or a mixture of chitinous and soft parts. For

TABLE 2

Prospective fate of pulsating regions in male genital discs

n	Source	Product after metamorphosis		
		Mostly soft parts	Equal soft and chitinous parts	Mostly chitinous parts
21	Pulsating region of a disc	18	2	1
39	Non-pulsating region of a disc	4	15	20

n = number of metamorphosed implants. Soft parts are paragonia, sperm pump, ejaculatory duct; chitinous parts are anal plates, claspers, lateral plates, penis apparatus.

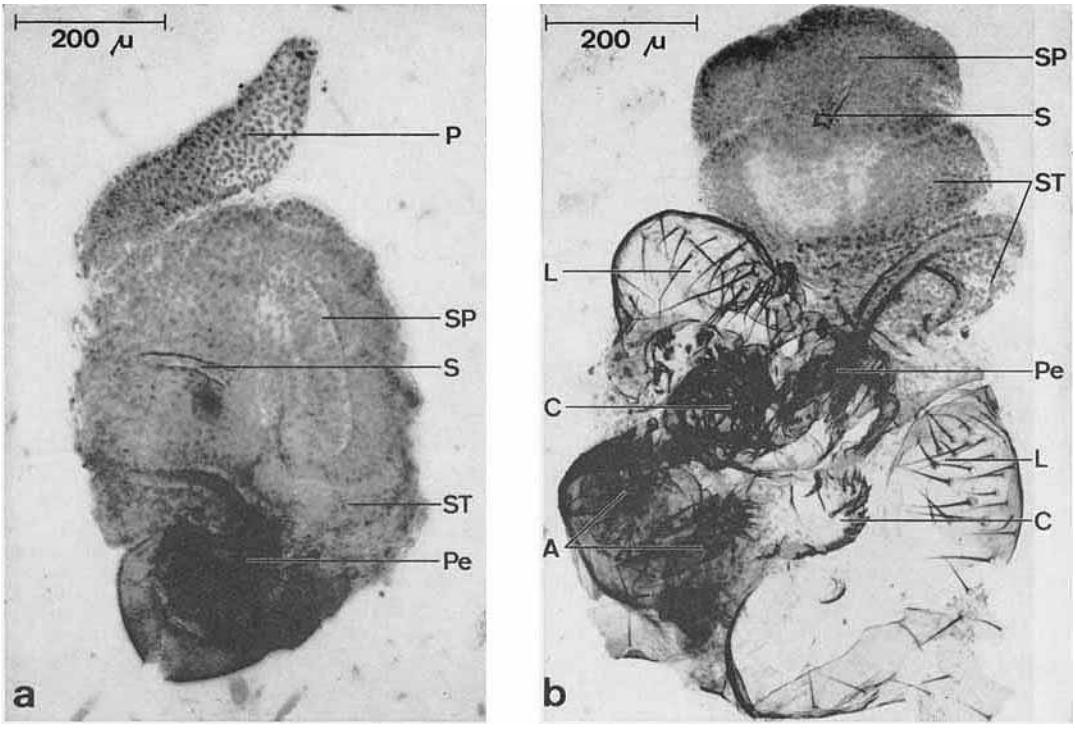

Fig. 2 Photographs showing chitinous and soft parts from pulsating (a) and non-pulsating (b) discs. A, anal plates; C, claspers; L, lateral plates; P, paragonio; Pe, penis apparatus; S, sclerite of sperm pump; SP, sperm pump; ST, unidentified soft tissue.

a description of the genital apparatus see Ursprung, 1959.

Origin of the contracting cells. As a corollary to the above experiment one would like to know whether the contracting regions originated within a particular portion of the disc. It was most logical to test the area of the disc whose cells are determined for soft parts. An anlage plan for the male genital disc (Hadorn et al., '49) locates the soft parts primarily in the anterior half of the disc. This finding was confirmed in a control experiment in which the posterior and the anterior halves contained cells determined for chitinous structures, while only the anterior half contained the cells determined for the paragonia, ductus and sperm pump. Based on this information we cut the discs into anterior and posterior halves. Twenty-four anterior and 24 posterior halves of male genital discs from mature larvae (114–116 hours) were implanted into young adults. Twenty-one (88%) of the anterior halves pulsated, while only two (8.3%) of the posterior halves did so.

DISCUSSION

The occurrence of pulsations in cultured male genital discs had escaped detection in previous work, although it occurs with a repeatable frequency under certain experimental conditions. Not all imaginal discs of *Drosophila* have this ability, however. Our experiments show that leg and wing discs do not pulsate and that female genital discs pulsate only rarely. On the other hand, pulsations have been observed in cultured thoraric imaginal discs of the mosquito, *Aedes aegypti* (Chabaud et al., '60), and in cultured leg discs of *Culex pipiens* (W. Spinner, pers. comm.). Since only one type of disc (the genital disc) pulsated in our experiments it is unlikely that the contractions were caused by invading cells of the adult host. Furthermore, no instance of pulsations has been found in cultures of young male genital discs even after continuous culture for several years (Hadorn, '63, '66), whereas mature male genital discs pulsate after only eight days in culture. From all this we conclude that the cells which contract are those of the implant, and do not come from the host.

The parameters that we found to be important in the differentiation of pulsating genital discs were donor age, host age, and the presence of active ring glands. These are the same factors that Schläpfer ('63) reported as affecting the production of eye pigment in cultured eye discs.

The fact that pulsations did not appear in cultured male genital disc fragments until 8 to 14 days after implantation suggests that the final steps in the formation of the contracting region are independent of the hormones which control adult metamorphosis. However, this does not mean that the percentage of pulsating discs produced is unaffected by the culture milieu. For example, young hosts provided a considerably better environment than old hosts for the realization of pulsating discs. Is it possible that the young hosts have residual ecdysone that is no longer present in the old hosts, as Shaaya and Karlson ('65) found for *Bombyx mori*? Unfortunately, we lack information on the ecdysone content of *Drosophila* adults. However, support for the view that hormones can influence the occurrence of pulsations comes from the experiment with implanted ring glands, in which the percentage of pulsating discs formed in both young and old hosts was markedly improved by the addition of ring glands from mature larvae. Secondly, it is significant that young discs were less able to differentiate pulsating regions than mature discs after *in vivo* culture. Clearly, the state of competence of the male genital disc to differentiate pulsating regions without metamorphosis changes during the 20 hours preceding pupation, presumably under hormonal control. Perhaps it is because mature discs (114–120 hours) are so close to pupation that such a high percentage (about 80%) pulsate after culture *in vivo*. This would also explain why the percentage for mature discs was affected only slightly by the addition of ring glands, whereas the percentage of cultured young discs which pulsated was increased greatly by this treatment. We concluded, then, that the differentiation of the contracting region in the male genital disc is initiated in some way in the larva, probably by ecdysone, and may proceed in the absence of further ecdysone, although the percentage of

discs which do differentiate in this manner is higher in the continued presence of active ring glands.

Because the extent of proliferation in the cultured genital discs had no effect on the percentage of pulsating implants found, we conclude that the production of new cells is not necessary for the differentiation of the contracting region and that original cells of the implant have this ability. Are all of the cells of the disc able to contribute to the pulsating region? Apparently not, since only a small portion of a disc ever pulsates. The obvious question at this point is whether the prospective fate of those cells which differentiate contracting regions is different from the prospective fate of those cells which do not. The first clue that there is a relation between the pulsating region and normal metamorphosed structures comes from the observation that the female genital discs pulsated only rarely, as compared to the high frequency for male genital discs. While several structures of the male genital apparatus contract in adult life (ejaculatory duct, sperm pump and paragonia), only one small part of the female genital apparatus contracts, the spermathecae (G. Mindek, pers. comm.). However, there is also direct evidence that the contracting region of the cultured genital disc is the same region that produces contracting soft parts after metamorphosis. An examination of the whole mounts of metamorphosed pulsating and non-pulsating portions of cultured discs showed that the prospective fate of the pulsating region was primarily contractile soft structures, while the non-pulsating fragments made primarily chitinous structures. Furthermore, the experiment with cultured anterior and posterior halves clearly demonstrates that only those fragments which contained cells determined for soft parts could form pulsating regions.

Thus, we have discovered a situation in which a normal physiological step in adult differentiation occurs in isolation without accompanying morphological changes. The formation of pulsating regions apparently takes place out of sequence, since our dissections of metamorphosing pupae showed that morphological differentiation *in situ* precedes the onset of contractility. This system may prove useful in studying the control of biochemical differentiation in multicellular organisms.

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