INDUCED OVARIAN DEVELOPMENT IN DECAPITATED MOSQUITOES BY TRANSFUSION OF HAEMOLYMPH

By J. D. GILLETT

East African Virus Research Institute, Entebbe, Uganda

(*Received* 21 *March* 1958)

Ovarian development in mosquitoes appears to be under hormonal control. Evidence for this view has been derived from experiments involving removal of the endocrine organs assumed to be responsible for production of the hormone by decapitation or ligation of the thorax, before and after a critical period. (Detinova, 1945; Clements, 1956; Gillett, 1956*b*, 1957). Such evidence, however, even when supported by histological studies, is indirect and does not entirely exclude the possibility of alternative explanations; nor are the grounds for criticism removed when insects are joined in parabiosis (Wigglesworth, 1936, 1954; Williams, 1946). The present paper describes induction of ovarian development in mosquitoes, decapitated before the critical period, by transfusion of haemolymph from normal donors.

MATERIALS, METHODS AND TERMS

Mosquitoes

A strain of *Aëdes* (*Stegomyia*) *aegypti* (L.) originating from Lagos, Nigeria, and colonized in Entebbe for 10 years, was used in all the experiments. This strain was used also in previous experiments on the hormonal control of ovarian development (Gillett, 1956*b*, 1957). Each day female pupae were isolated in separate tubes within 5 hr. of pupation, by the 'dry' method described by Gillett (1956*a*); these were divided into three groups (test donors, test recipients and controls) and placed in an incubator at 28° C. Adults emerged 2 days later (day 0) in their separate tubes, and were fed on day 3.

Feeding. The period during which hormone is assumed to be released varies from mosquito to mosquito. This variation, however, can be decreased if the mosquitoes are not given fruit juice before the blood-meal (Gillett, 1957). It seems possible that previous water feeding may act in the same way as fruit feeding, the amount of water or fruit juice taken up by a mosquito being variable and equally difficult to control. In these experiments fruit feeding was excluded, and water feeding reduced to a minimum by the method of allowing adults to emerge on barely moist paper. Feeding, therefore, refers to blood meals only.

The mosquito's own blood, as distinct from ingested blood in the stomach, is referred to as haemolymph.

Treatment

In a previous experiment groups of fruit-starved mosquitoes reared from 'dry' pupae were decapitated at intervals after a feed, and were later dissected to see what proportion had developed their oocytes to maturity (Gillett, 1957). In the figure, which is based on these previous findings, it is shown that after 4 hr. the proportion rises steeply, and it is assumed that release of hormone usually occurs between 4 and 8 hr. after a feed. Nevertheless, it seemed possible that hormone, once released, might be rapidly inactivated, and thus that there might be a better chance of obtaining haemolymph with active hormone in the earlier half of this period, that is, between 4 and 6 hr. Similarly, it seemed possible that the oocytes might not enter a receptive state until shortly before, or at the time of, hormone release. Thus the optimal time for transfusion might be supposed to be between 4 and 6 hr. for the donors (50% of which might be expected to contribute hormone), and between 2 and 4 hr. for the recipients (8% of which might be expected to develop their oocytes to maturity independent of treatment). For convenience the period 4–6 hr. is referred to as the *active period*, and the period 2–4 hr. as the *receptive period*, although these two periods are likely to overlap in any individual mosquito.

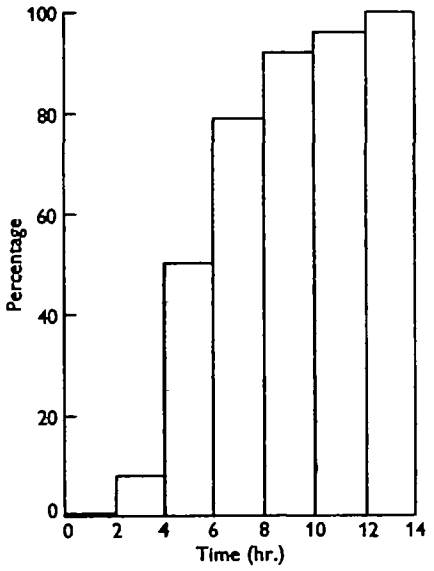

Fig. 1. Proportion of decapitated *A. aegypti* developing mature oocytes 48 hr. after a feed. Ordinate: percentage with mature oocytes (groups of 24). Abscissa: interval between feed and decapitation

Test mosquitoes were chilled for 100 sec. during the receptive period (the intervals between feeding and treatment being shown in each experiment) and decapitated, the wound being sealed with paraffin wax. About 0·2 mg. haemolymph was then withdrawn by thoracic puncture with a glass micropipette of 50–80 μ

external diameter. Each of these *recipient* mosquitoes was then chilled again for 30 sec. and transfused through the same hole with about the same quantity of haemolymph, drawn from the thorax of a chilled *donor* mosquito, which had been fed 2 hr. before the recipient. Immediately after transfusion the donor was decapitated and the wound sealed with wax. Recipients and donors were then kept at 28° C. until day 5 (2 days after treatment), when both were sacrificed and dissected for ovarian inspection. The technique for thoracic puncture was very much simpler than that described by Shambaugh (1952) or by Weathersby (1952), although the dimensions of the micropipettes were based entirely on the figures given by Weathersby, which were confirmed by trial as being optimal. The technique was based on a method regularly used by Dr Loring Whitman of the Rockefeller Foundation Laboratories, New York, for the intrathoracic injection of mosquitoes, with virus.

Control mosquitoes were taken in pairs on the same day and at the same interval after being fed as were the test recipients in each trial. After chilling, each partner was decapitated and sealed with wax immediately before reciprocal transfusion. Thus each control mosquito became both donor and recipient, as the haemolymph was exchanged between pairs.

Response

For *A. aegypti* it has been shown that, whereas in mosquitoes decapitated before the critical period ovarian development stops at, or before Christophers's (1911) stage 3, in those decapitated after the critical period development goes on to maturity (stage 5). Thus there is presumptive evidence that the hormone is concerned only in development from stage 3 to maturity (Gillett, 1956*b*). Response to treatment, therefore, was regarded as positive only if mature oocytes were found when the mosquitoes were dissected on day 5.

The techniques used resulted in some premature deaths. In order to prevent any unconscious selection of data it was decided at the outset that each trial in each experiment should be continued until a predetermined number of mosquitoes had survived 48 hr. following treatment. Thus in Exp. 1 each of six trials was continued until 50 test recipients and 50 controls had survived for the requisite period, and Exp. 2 was continued until 100 test donor-recipient pairs had survived.

EXPERIMENTS

Two main experiments were carried out, both being based on the assumption that hormone produced in the head (presumably from the neurosecretory cells of the brain) either exerts its effect by being released directly into the haemolymph, or by activating the corpus cardiacum or corpus allatum which are situated in the thorax (Bodenstein, 1945; Scharrer, 1952, Thomsen, 1952; Gillett, 1956*b*). The plan was to see if haemolymph from donors, presumed to contain hormone, would induce ovarian development to maturity when transfused to mosquitoes deprived of the capacity to produce their own hormone. It was clear, however, that by taking recipients and controls in the receptive period, some of them would in any case develop oocytes to maturity; the question, therefore, was whether or not the response rate would be higher in test recipients than in the controls.

Experiment 1. As the best time for treatment was not known, it was decided first to take recipients early in the receptive period and to transfuse them with haemolymph from donors early in the active period; then to take recipients late in the receptive period and to transfuse them from donors late in the active period. Having thus straddled the target, intermediate times were chosen in a series of trials in the hope of finding the optimal time both for recipients and donors.

Table 1. *Effect of haemolymph transfusion on development of the ovaries in decapitated* Aëdes aegypti

Trial no.	Test mosquitoes			Control mosquitoes		
	Interval (hr.)* between feed and transfusion		Proportion of recipients with mature oocytes 48 hr. after feed	Interval (hr.)* between feed and transfusion		Proportion of recipients with mature oocytes 48 hr. after feed
	Donor	Recipient		Donor	Recipient	
1	4	2	4/50	2	2	3/50
2	4½	2½	14/50	2½	2½	5/50
3	4½	2½	9/50	2½	2½	6/50
4	5½	3½	9/50	3½	3½	6/50
5	5½	3½	9/50	3½	3½	4/50
6	6	4	20/50	4	4	17/50
Total			65/300			41/300

* ±2½ min.

The results, arranged in order of increasing time interval between feeding and treatment, are shown in Table 1. It will be noted that the time interval in trials 2 and 3 was the same. The reason for this was that the results of trial 2 showed a difference in response rate between test recipients and controls which was significant at the 2·5% level (χ^2 for 1 degree of freedom = 5·263, $0{\cdot}02 < P < 0{\cdot}025$), and it was felt that if this result was attributable to the treatment, then it should be reproducible. The results of trial 3, however, showed less difference in response rate between test recipients and controls, and, in fact, in none of the trials which followed was such a difference recorded. Nevertheless, in all six trials the difference was in the expected direction, a result which is significant at the 1% level in a one-sided test concerning increase and not merely change in response rate (t for 5 degrees of freedom = 3·554, $0{\cdot}005 < P < 0{\cdot}01$). The over-all picture is, of course, largely influenced by the result of trial 2, which increases the mean difference in response rate, but at the same time increases the standard deviation. If this trial is excluded the results are significant at the 0·5% level for a one-sided test (t for 4 degrees of freedom = 4·743, $0{\cdot}001 < P < 0{\cdot}005$). It seems, therefore, that transfusion of haemolymph to recipients between 2 and 4 hr. after a feed, from donors fed 2 hr. earlier, results in an increase in response rate over those transfused by donors fed at the same time.

So far consideration has been given to difference in response rate in test recipients and controls, irrespective of the fate of the oocytes in the donors. Actually, these

donors were decapitated immediately after contributing haemolymph in order to see if they had, in fact, entered the period of hormone production by the time treatment was given. But while each trial was continued until 50 recipients and 50 controls had survived, no such precautions were necessary with the donors; if a recipient survived for 48 hr. following treatment, the result was scored whether the donor survived or not; if, on the other hand, a recipient died prematurely, a second recipient was transfused by a second donor, no matter what the fate of the first donor happened to be. Thus, while all recipients and controls had a denominator of 50 in each trial, the number of surviving donors that could be scored varied from trial to trial. The results are given in Table 2 and show a steady increase in the proportion of donors which went on to develop mature oocytes from 38% at 4 hr. to 80% at 6 hr.

Table 2. *Effect of haemolymph withdrawal and decapitation on development of the ovaries in* Aëdes aegypti

Trial no.	Interval (hr.)* between feed and decapitation	Proportion of mosquitoes with mature oocytes 48 hr. after feed
1	4	11/29 (38%)
2	4½	35/62 (57%)
3	4½	25/43 (58%)
4	5½	20/34 (59%)
5	5½	24/33 (73%)
6	6	28/35 (80%)
Total		143/236 (61%)

* ±2½ min.

Experiment 2. About one-quarter of all donors and recipients failed to survive treatment. It followed, therefore, that the chance of the two partners in a single donor-recipient pair both surviving was only about one in two. Nevertheless, it was decided to study the response rate in donor-recipient pairs, discarding all results except when donor and recipient both survived. The question, then, was whether the response rate in recipients of haemolymph from positive donors was higher than that in recipients of haemolymph from negative donors.

As it was essential to have negative as well as positive donors it was decided to take mosquitoes at 4½ hr. after a feed (see Table 2), and to transfuse their haemolymph to decapitated recipients at 2½ hr. after a feed. The choice of these time intervals not only ensured that there would be a large number of negative donors, but allowed all surviving donor-recipient pairs from trials 2 and 3 of Exp. 1 to be incorporated into the results. All donors were decapitated immediately after treatment. The results, given in Table 3, do not show a significant difference in response rate between recipients of haemolymph from positive donors and those from negative donors, nevertheless, the difference is again in the expected direction, the response rate of one being double that of the other.

Table 3. *Effect of haemolymph transfusion from positive and negative donors on development of the ovaries in decapitated* Aëdes aegypti.

Proportion of recipients developing mature oocytes following transfusion		Total
From positive donors	From negative donors	
14/63 (22%)	4/37 (11%)	18/100
χ^2 for 1 degree of freedom = 1·356*, 0·10 < P < 0·125 (for one-sided test)		

* Yates's correction applied.

DISCUSSION

The use of transfusion methods to demonstrate the presence of hormones in the haemolymph of insects has met with varying success (Fraenkel, 1935; Fukuda, 1938; Mokia, 1941; Meyer, 1953; Hasegawa, 1957). In theory it would appear to offer the final proof that hormonal mechanisms are involved, but in practice there may be too many unknown factors to allow clear cut results.

In the mosquito *A. aegypti* it has been assumed, as a working hypothesis, that some time between 4 and 6 hr. after a feed hormone released from the head (presumably from the neurosecretory cells of the brain) is present in the haemolymph, and normally results in the development of activated oocytes from stage 3 to maturity. Thus, if haemolymph is withdrawn from a donor mosquito during this period and transfused to a mosquito which has been deprived of the capacity to produce its own hormone, normal development of the ovaries might be expected to follow in the recipient. It is not known, however, whether hormone, once released, remains free for some time in the haemolymph, or whether, even if produced over a long period, it is inactivated almost as fast as it is produced. Nor is it known whether the receptor organs (for example, other endocrine organs or perhaps the oocytes themselves) enter a receptive period of limited duration, or whether they require a constant supply of freshly liberated hormone over a prolonged period.

The chances, then, of taking haemolymph from a donor at the optimal time and transfusing it into a recipient also at the optimal time might be very slender indeed. Better results might have followed had repeated transfusions been given to each recipient. This possibility was realized at the time, but it was felt that mosquitoes are not sufficiently robust to withstand such treatment. Better results might also have been obtained had donors and recipients been fed at the same time; thus while the recipients would have been decapitated between 2 and 4 hr. after the feed, transfusion would have been postponed for a further 2 hr. This was, in fact, tried, but as it necessitated extra manipulation it was abandoned in favour of the technique described.

Besides these and other unknown physiological factors, there exist possible indirect effects of the necessary manipulative treatment. That chilling and the

act of decapitation itself do not adversely affect the response seems clear from the results shown in Table 2 and those of many other experiments on this species. One must, however, consider the possible effects of puncture and of temporary reduction in haemolymph volume between withdrawal and replacement by fresh haemolymph. In these experiments haemolymph withdrawn from donors was not replaced, yet 61 % (143/246) developed oocytes to maturity after decapitation (Table 2). This is not significantly different from the proportion of 12/24 obtained previously by Gillett (1957) following simple decapitation between 4 and 6 hr. after a feed (χ^2 for 1 degree of freedom = 1·015, $0{\cdot}30 < P < 0{\cdot}50$).

It is well known that changes occur in haemolymph on exposure to air, which are manifested by darkening (Dennell, 1947; Fraenkel & Rudall, 1947). This change takes 90–120 sec. in *A. aegypti*, but as the interval between withdrawal of haemolymph from a donor to transfusion to a recipient was only 20–40 sec. this change did not occur in practice. The amount of haemolymph exchanged was 0·2 mg., but as the average weight of unfed *A. aegypti* is only 1·32 mg. (Roy, 1936), this represents a substantial proportion (15 %) of the body weight.*

That apparently minor differences in technique may have far-reaching effects on the results is shown by the difference in response between fruit-fed and fruit-starved mosquitoes, and emphasizes the need for caution when comparing results.

It has been shown that while initiation of ovarian development in *A. aegypti* is apparently independent of endocrine organs in the head (Clements, 1956; Gillett, 1956*b*), development stops at or before stage 3 unless the head is left intact for a period of 4–8 hr. following the feed (Gillett, 1956*b*, 1957). If, however, groups of fed normal *A. aegypti*, kept at 28° C., are sacrificed and dissected at hourly intervals, it is found that further development of stage 3 oocytes does not occur until about 14 hr. after the feed. This perhaps supports the possibility, which has already been mentioned, that the hormone from the head serves in turn to activate other endocrine organs, which must lie posterior to the head (presumably in the thorax). While preliminary trials involving decapitation and extirpation of the anterior part of the thorax, and delayed transfusion between 8 and 14 hr. after the feed, have not so far supported this, it is hoped to be able to investigate the possibility further.

SUMMARY

1. The final stages of ovarian development in the mosquito *Aëdes aegypti* appear to be under the control of a hormone which is produced in the head.

2. Decapitation of groups of mosquitoes before a critical period prevents final development of the ovaries; decapitation after the critical period allows development of the ovaries to maturity.

3. Transfusion of haemolymph from donor mosquitoes in the middle of the critical period to recipient mosquitoes decapitated at a stage just before, or very early in the critical period, results in a higher number developing their oocytes to

* Mellanby (1939) has stated that the amount of haemolymph in insects varies from 15 to 70 % of the total body weight.

maturity than when transfusion is between donors and recipients at the same stage.

4. Similarly, transfusion from known positive donors (i.e. those which by decapitation and subsequent dissection were known to have already produced hormone at the time of transfusion) results in a higher number of recipients developing oocytes to maturity, than when transfusion is from known negative donors.

I am greatly indebted to Dr C. L. Chiang, Assistant Professor of Biostatistics, University of California, and to Dr Loring Whitman of the Rockefeller Foundation Laboratories, New York: the former for a most helpful discussion of the results and for pointing out the use of the one-sided test for handling the figures; and the latter for the privilege of seeing his simplified microinjection technique at first hand during a Rockefeller Foundation Fellowship in 1955–56.

REFERENCES

BODENSTEIN, D., (1945). The corpora allata of mosquitoes. *Bull. Conn. Agric. Exp. Sta.* no. 488, pp. 396–405.

CHRISTOPHERS, S. R. (1911). The development of the egg follicle in anophelines. *Paludism*, **2**, 73–88.

CLEMENTS, A. N. (1956). Hormonal control of ovary development in mosquitoes. *J. Exp. Biol.* **33**, 211–23.

DENNELL, R. (1947). A study of an insect cuticle: the formation of the puparium of *Sarcophaga falculata* Pand. (Diptera). *Proc. Roy. Soc.* B, **134**, 79–110.

DETINOVA, T. S. (1945). On the influence of glands of internal secretion upon the ripening of the gonads and the imaginal diapause in *Anopheles maculipennis*. (In Russian with English summary.). *Zool. Zh.* **24**, 291–8.

FRAENKEL, G. (1935). A hormone causing pupation in the blowfly *Calliphora erythrocephala*. *Proc. Roy. Soc.* B, **118**, 1–12.

FRAENKEL, G. & RUDALL, K. M. (1947). The structure of insect cuticles. *Proc. Roy. Soc.* B, **134**, 111–43.

FUKUDA, S. (1938). Note preliminaire sur la transformation de la peau larvaire en puparium chez les larves de *Sturmia sericariae*. *Proc. Imp. Acad. Japan*, **13**, 114–16.

GILLETT, J. D. (1956*a*). Genetic differences affecting egg-laying in the mosquito *Aëdes* (*Stegomyia*) *aegypti* (Linnaeus). *Ann. Trop. Med. Parasit.* **50**, 362–74.

GILLETT, J. D. (1956*b*). Initiation and promotion of ovarian development in the mosquito *Aëdes* (*Stegomyia*) *aegypti* (Linnaeus). *Ann. Trop. Med. Parasit.* **50**, 375–80.

GILLETT, J. D. (1957). Variation in the time of release of the ovarian development hormone in *Aëdes aegypti*. *Nature, Lond.*, **180**, 656–7.

HASEGAWA, K. (1957). The diapause hormone of the silkworm, *Bombyx mori*. *Nature, Lond.*, **179**, 1300–1.

MELLANBY, K. (1939). The function of insect blood. *Biol. Rev.* **14**, 243–60.

MEYER, J. H. (1953). Die Bluttransfusion als Mittel zur Überwindung letaler Keimkombination bei Lepidopteren-Bastarden. *Z. wien. ent. Ges.* **38**, 41–80.

MOKIA, G. G. (1941). Contribution to the study of hormones in insects. *C.R.* (*Doklady*) *Sci. U.R.S.S.* **30**, 371–3.

ROY, D. N. (1936). On the role of blood in ovulation in *Aëdes aegypti* Linn. *Bull. Ent. Res.* **27**, 423–9.

SHAMBAUGH, G. H. (1952). Microinjection technique in mosquitoes. *Mosquito News*, **12**, 216.

SHARRER, B. (1952). Neurosecretion. XI. The effects of nerve section on the intercerebralis-cardiacum-allatum system of the insect *Leucophaea maderae*. *Biol. Bull., Woods Hole*, **102**, 261–72.

THOMSEN, E. (1952). Functional significance of the neuro-secretory brain cells and the corpus cardiacum in the female blow-fly, *Calliphora erythrocephala* Meig. *J. Exp. Biol.* **29**, 137–72.

WEATHERSBY, A. B. (1952). The role of the stomach wall in the exogenous development of *Plasmodium gallinaceum* as studied by means of haemocoel injection of susceptible and refractory mosquitoes. *J. Infect. Dis.* **91**, 198–205.

WIGGLESWORTH, V. B. (1936). The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Quart. J. Micr. Sci.* **79**, 92–121.

WIGGLESWORTH, V. B. (1954). *The Physiology of Insect Metamorphosis*. Monographs in Exp. Biol. No. 1. Cambridge University Press.

WILLIAMS, C. M. (1946). Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull., Woods Hole*, **90**, 234–43.