Distribution of Mercury in the Eggs of the False Melon
Beetle, *Atrachya menetriesi* FALDERMANN (Coleoptera :
Chrysomeridae), Treated with Mercuric
Chloride for Breaking Diapause

Yoshikazu ANDO¹

Laboratory of Applied Entomology, Faculty of Agriculture, Iwate University, Morioka, 020 Japan

(Received January 20, 1971)

Mercuric chloride was effective in breaking diapause in the egg of *Atrachya menetriesi*. The distribution of mercury in treated eggs was examined quantitatively. To break diapause eggs were dipped in an aqueous solution of mercuric chloride (0.125mM) for 5 min. About 20 μ g of mercury per 100 mg of eggs was found in the chorion and none in other parts. Only a negligible amount was found in the larvae that hatched from treated eggs. Almost all the mercury was removed when eggs were washed in a dilute solution of cysteine hydrochloride or glutathione (reduced).

It seems that breaking of diapause is effected even when mercury does not penetrate the chorion.

INTRODUCTION

There are many insects which enter diapause at early embryonic stages (LEES, 1955). Hypotheses on the mechanism of breaking diapause in eggs have been proposed by several investigators (BODINE, 1932; SLIFER, 1946; HINTON, 1953; WAY, 1960; BROWNING, 1969). There is, however, no firmly established theory about the direct mechanism which keeps the embryo at the diapause stage. This is true even in *Bombyx mori*, in which the endocrine mechanism of the determination of diapause has been elucidated (FUKUDA, 1951; HASEGAWA, 1952). As reported in certain insects (SLIFER, 1946; HOGAN, 1961), egg diapause may sometimes be broken by chemicals. The utilization of chemicals may be one of the most efficient methods to solve the mechanism of breaking of an egg diapause.

The false melon beetle, *Atrachya menetriesi* FALDERMANN, deposits eggs in summer. Most of the eggs enter diapause at an early embryonic stage (MIYA, 1965). Diapause continued for a long time when they were kept at a high temperature (MAKI and ANDO, 1967). The diapause was easily broken by treatments with various mercury compounds and this effect might be caused by mercury ion itself (KURIHARA and ANDO, 1969). In order to elucidate the site and nature of this action, it is at first necessary to know whether or not mercury ion penetrates into the egg through the chorion. This paper reports an investigation of the distribution of mercury in eggs after treatment with mercuric chloride.

¹ Present address: Laboratory of Applied Entomology, Faculty of Agriculture, Hirosaki University, Hirosaki, 036 Japan.

MATERIALS AND METHODS

Adult beetles were raised throughout the year from chilled eggs. About equal number of both sexes of beetles were reared in glass vessels on fresh leaves of ladino clover at about 25°C. Many eggs were collected every day from this culture and held at 25°C. Diapausing eggs which had been kept for 30 to 50 days after deposition were dipped in aqueous solution of mercuric chloride in a cabinet controlled at $25\pm 1^\circ\text{C}$. They were then washed in distilled water for 10 min. One hundred mg of eggs were used for each analysis and 150 eggs were kept at 25°C for 20 days to determine the percentage hatching. The treated eggs were preserved under moist conditions at all times.

The digestion of the samples and the determination of mercury were carried out according to the method of UKITA *et al.* (1963), which is a modification of the official method of A.O.A.C. (1960) applied to the digestion of animal materials. In this experiment, 100 mg of eggs (739 ± 15 individuals) at a time was ground in a mortar. The sample was poured with a small quantity of distilled water into a digestion flask, then 10 ml of a mixture of sulphuric-nitric acid (1 : 1) and 100 mg of selenium powder were added as a digestion catalyzer. The flask was then connected to a special digestion apparatus (A.O.A.C., 1960) and heated continuously for about 2 hr. The mercury vapour produced by heating was led to a gas absorption tube. Thereafter, the digested material was filtered through paper after cooling. The residue was washed by 4 ml of dilute sulphuric acid and 10 ml of distilled water. The filtrates were poured into a flask and boiled again for about 2 hr after addition of 10 ml of 0.3M potassium permanganate. Thereafter, the digested material was cooled and 5 ml of 9M hydroxylamine hydrochloride was added to make it colourless. Finally, the digested solution was adjusted to a volume of 150 ml by adding distilled water.

The sample was transferred to a separating funnel and 10 ml or more of dithizone chloroform (6 mg dithizone dissolved in 1 l chloroform) was added and shaken vigorously for 1 min. The chloroform layer was drained to a second separating funnel containing 50 ml of 0.25 N sulphuric acid, and 10 ml of 5 M potassium bromide was added to it and shaken 1 min. The chloroform layer was removed subsequently and the aqueous layer was rinsed with 10 ml of chloroform. Next, a buffer solution (Na_2HPO_4 , 150 g, and Na_2CO_3 , 38 g dissolved in 1 l of water) was added to adjust the pH to about 6. Finally, mercury in the solution was extracted with 10 ml or more of dithizone chloroform and the transmittance was determined at 490 m μ with a spectrophotometer.

A standard curve was obtained as follows. Two, 4, 6, 8 and 10 μg (as Hg) of mercuric chloride were added to a separating funnel with an aqueous solution of 50 ml 0.25 N sulphuric acid, and then the added mercury was extracted as described above. All reagents used were of analytical grade.

RESULTS

Recovery Percentage of Added Mercury

Since diapause is broken by dipping eggs in a very dilute solution of mercuric chloride (KURIHARA and ANDO, 1969), a method of microanalysis is needed to detect the distribution of small quantities of mercury in the treated eggs. To test the efficiency of the method 10 ml each of six different concentrations of mercuric chloride

Table 1. PERCENTAGE RECOVERY OF MERCURY

Added mercury	Detected mercury	Recovery
5 μ g	4.71 μ g	94.2%
10	9.39	93.9
25	23.75	95.0
50	48.28	96.6
100	98.75	98.8
200	201.00	100.5

(5-200 μ g Hg in 10ml solution) were added to 100 mg of ground eggs, and the amount of mercury was measured by the above procedure.

As shown in table 1, the percentage recovery was higher than 90% in every trial, and it tended to be higher with the increasing amount of added mercury.

Relation between Dipping Time and Mercury Content of Eggs

Diapausing eggs were dipped in a solution of mercuric chloride (0.125 or 1.25 mM) for varying periods of time, washed in distilled water for 10min and their mercury content determined. The results are summarized in tables 2 and 3.

The mercury content of eggs increased with increasing the time of dipping in the solution. When eggs were dipped in a solution of 0.125 mM for 5min—a very effective treatment to break diapause—19.83 μ g of mercury was found in 100 mg of eggs. The concentration of mercury in the treated eggs was thus about 8 times higher than in the solution. The rate of hatching attained its maximum of 79.3% at 5 min ex-

Table 2. RELATION BETWEEN THE DIPPING TIME IN AN AQUEOUS SOLUTION OF MERCURIC CHLORIDE (0.125 mM) AND MERCURY CONTENT OF EGGS

Dipping time	Mercury found in eggs (μ g/100mg)	Percentage hatching
	Mean $\pm$ S.D.	
0 min	0.18 $\pm$ 0.13	0
1	11.55 $\pm$ 3.02	17.3
5	19.83 $\pm$ 3.46	79.3
30	44.37 $\pm$ 7.82	75.3
2 hr	74.72 $\pm$ 11.51	34.7
8	162.04 $\pm$ 23.87	0

Table 3. RELATION BETWEEN THE DIPPING TIME IN AN AQUEOUS SOLUTION OF MERCURIC CHLORIDE (1.25 mM) AND MERCURY CONTENT OF EGGS

Dipping time	Mercury found in eggs (μ g/100mg)	Percentage hatching
	Mean $\pm$ S.D.	
10 sec	37.80 $\pm$ 7.01	43.3
1 min	86.25 $\pm$ 15.28	21.3
5	151.51 $\pm$ 26.64	0
30	340.74 $\pm$ 31.92	0

posure to 0.125 mM solution. It decreased again when eggs were dipped for more than 2 hr because of high mortality.

A similar trend was obtained when the eggs were dipped in a solution of 1.25 mM, although the mercury content of the treated eggs was far more than in the previous test even when the dipping time was the same. This experiment decreased the hatchability, compared with the previous one, even when the same amount of mercury was detected in the eggs. No larvae hatched from eggs dipped for 5 or 30 min in a 1.25 mM solution. The eggs were killed at the diapause stage.

Distribution of Mercury in the Treated Eggs

Although mercury is highly toxic to most organisms, many larvae hatched normally from the eggs treated with a moderate concentration of mercuric chloride. A question arises therefore as to whether mercury remains on the egg surface or penetrates through the chorion. The following experiment was done to examine the presence or absence of mercury in different parts of the eggs after treatment.

Eggs treated with mercuric chloride were fixed with CARNOY's fluid over night. Thereafter, the eggs were divided into two parts; the chorion and the egg contents other than the chorion. That is, the eggs were hulled with fine forceps carefully in distilled water under a binocular dissecting microscope, and the mercury content of each part was assayed (Table 4).

Only a negligible amount of mercury was found in the egg contents other than the chorion immediately after the eggs had been treated with mercuric chloride (0.125mM) for 5 min. On the other hand, 17.07 μ g mercury was detected from the chorion. After the eggs were removed from their chorion, their mercury content did not differ significantly from that of untreated eggs. Since it seemed possible that

Table 4. DISTRIBUTION OF MERCURY IN THE EGGS WHICH WERE DIPPED IN A SOLUTION OF MERCURIC CHLORIDE

Concentration of HgCl ₂ (mM)	Dipping time (min)	Mercury found (μ g/100mg)	
		In chorion	In egg contents other than chorion
		Mean $\pm$ S.D.	Mean $\pm$ S.D.
0.125 ¹	5	17.07 $\pm$ 3.04	0.15 $\pm$ 0.11
0.125 ²	5	16.54 $\pm$ 2.54	0.20 $\pm$ 0.16
1.25	30	297.13 $\pm$ 30.36	25.30 $\pm$ 4.82
Control (untreated)	—	0.15 $\pm$ 0.06	0.12 $\pm$ 0.11

¹ Analysis was carried out immediately after treatment.

² Analysis was carried out just before hatching.

Table 5. MERCURY CONTENT OF LARVAE THAT HATCHED FROM THE EGGS TREATED WITH MERCURIC CHLORIDE (0.125 mM) FOR 5 MIN

Treatment	Mercury found (μ g/739 individuals)	
	In cast-off chorion	In newly hatched larvae
	Mean $\pm$ S.D.	Mean $\pm$ S.D.
Mercuric chloride	15.83 $\pm$ 2.63	0.18 $\pm$ 0.13
Low temperature	0.14 $\pm$ 0.10	0.15 $\pm$ 0.12

mercury might penetrate through the chorion several days after the treatment, the distribution of mercury was again examined just before hatching. At this time the eggs had increased their volume considerably as a result of water uptake. The result of analysis showed, however, no substantial increase in the amount of mercury in the egg contents.

On the other hand, a considerable amount of mercury was found not only in the chorion but also in the contents of the eggs when the eggs were dipped in a solution of higher concentration (1.25mM) for 30 min. As might be expected, all eggs were killed by such a treatment.

Furthermore the larvae that hatched from the eggs treated with mercuric chloride showed no difference in their mercury content from those which hatched from chilled eggs (Table 5). It was observed that hatching larvae bit through the chorion, but did not swallow it.

Effect of Sulfhydryl Compounds on Eggs Previously Treated with Mercuric Chloride

Three hundred mg of diapause eggs were dipped in a solution of mercuric chloride (0.125mM) for 5 min, and then washed in distilled water for 10 min. 100 mg of them were dipped three times in a 10 ml solution of cysteine hydrochloride for 10 min, and another 100 mg dipped in a solution of glutathione (reduced). The remaining 100 mg were dipped in distilled water. The mercury content was determined for each of these three groups of eggs as well as for the solution in which they were washed.

Almost all the mercury was recovered from the aqueous solutions of sulfhydryl compounds, and only a negligible amount was detected from the eggs (Table 6). Thus, mercury seems to be easily combined with sulfhydryl compounds and removed

Table 6. EFFECT OF SULFHYDRYL COMPOUNDS ON THE EGGS TREATED WITH MERCURIC CHLORIDE (0.125 mM) FOR 5 MIN

Reagents	Concentration (mM)	Mercury found ($\mu\text{g}/100\text{mg}$)		Percentage hatching
		In eggs	In solution or reagent	
Cysteine hydrochloride	1	1.05	19.70	0.7
Glutathione (reduced)	1	0.85	16.82	0
Control (water)	—	20.86	0.92	76.7

Table 7. PERIOD OF TIME REQUIRED FOR BREAKING DIAPAUSE AFTER TREATMENT WITH MERCURIC CHLORIDE (0.125 mM) FOR 5 MIN

Time interval between mercury and cysteine hydrochloride treatments	Mercury found in the eggs ($\mu\text{g}/100\text{mg}$)	Percentage hatching
0 hr	1.05	0.7
1	1.38	4.0
2	2.06	13.3
3	1.92	26.7
4	0.97	74.0
6	2.08	78.0
20	1.76	80.7
Control (washing in water)	19.31	79.3

Table 8. DEVELOPMENT OF LARVAE THAT HATCHED FROM EGGS TREATED WITH MERCURIC CHLORIDE (0.125 mM)

	No. of examined	Egg treatment	
		Mercuric chloride	Low temperature
		Mean $\pm$ S.D.	Mean $\pm$ S.D.
Hatchability	450	78.4 $\pm$ 5.5	98.6 $\pm$ 0.7
Duration of larval and pupal stages in days (25°C)	30	25.6 $\pm$ 0.6	25.4 $\pm$ 0.7
Percentage of adult emergence ¹	200	83.5 $\pm$ 5.8	80.5 $\pm$ 6.2
Weight of adult (mg) ♀	20	18.78 $\pm$ 1.54	18.87 $\pm$ 1.98
♂	20	13.57 $\pm$ 1.46	13.75 $\pm$ 1.43

¹ No. of adult emergence/No. of full grown larvae.

almost completely from the chorion. After this treatment the eggs were still in diapause but remained alive. Most of the mercury remained in the eggs which were washed in distilled water.

By utilizing the mercury-removing action of sulfhydryl compounds, it was possible to determine the duration of time necessary for breaking diapause by mercury. The results of such an experiment are given in Table 7. In this experiment, eggs which had been treated with mercury were dipped at different times after treatment in a solution of cysteine hydrochloride. The mercury content of the eggs and the rate of hatching were examined in each treatment. It is clear that mercury was removed from the eggs when they were dipped in a solution of cysteine hydrochloride even when a long time had elapsed after the mercury treatment. The percentage hatching varied with the time interval between the mercury and sulfhydryl treatments. From this variation it seems that diapause was effectively broken when mercury remained in the chorion for at least 4 hr.

Development of Larvae that Hatched from Eggs Treated with Mercuric Chloride

The larvae that hatched from eggs treated with mercuric chloride developed quite normally compared with those obtained from chilled eggs, although the hatchability was lower in the former than in the latter (Table 8). No difference was found between them in the duration of larval development, mortality and weight of the adults. The beetles could be reared for 6 successive generations by means of breaking diapause with mercuric chloride. It seems that there was no harmful effect of mercuric chloride treatment at the diapause stage for future development.

DISCUSSION

HOGAN (1961, 1962, 1964) has discussed on the termination of diapause in the egg of *Teleogryllus commodus* by the application of urea. He considers that under natural conditions the synthesis of urea in the eggs during the winter leads to the termination of diapause. This supposition is based on the results of experiments that show that diapause is terminated promptly when a solution of urea is applied to the diapausing eggs. He proved by means of radioisotopes that the applied urea actually penetrated into the eggs (HOGAN, 1962). There is little doubt, however, that the breaking of diapause in the egg of *Atrachya* is effected without mercury penetrating through

the chorion. Thus, mercury could not be detected in parts other than the chorion after the eggs had been dipped in an aqueous solution of mercuric chloride. Also, no mercury was detectable in the larvae that hatched from the eggs treated with mercuric chloride. Furthermore, the applied mercury was easily removed from the eggs when they were washed in a solution of sulfhydryl compound. These facts may be interpreted as showing that the chorion itself is the site of action of mercury. Since it is quite improbable that mercury is accumulated in the egg during hibernation under natural conditions, mercury is not an indispensable factor for terminating diapause. It may act upon the chorion, and some change thereby induced in the chorion may lead to the breaking of diapause.

There are many species of insects in which the absorption of water is indispensable for the normal post-diapause development of their eggs. From this, a hypothesis has been proposed that the occurrence of a barrier for water absorption on the egg surface is the primary cause of diapause in *Melanoplus differentialis* (SLIFER, 1946). In this grasshopper, the diapause can be broken by immersing the eggs in xylol and other wax solvents. SLIFER thought that the treatment dissolved the waxy layer over the surface of the hydropyle, which prevents the entry of water needed to complete development. Consequently, xylol is effective in breaking diapause only after the cuticle and the wax layer over the general egg surface and the hydropyle area have already been secreted by the serosal cells. In *Atrachya* also, the absorption of water seems to be essential for post-diapause development. The non-diapausing egg remains in a state of quiescence unless it is in contact with moisture (unpublished data). The diapausing egg does not absorb water at all even when it is kept moist, but it begins to do so as a result of the treatment with mercuric chloride. Subsequently, its development proceeds rapidly. On the other hand, the egg of this beetle differs from the eggs of *Melanoplus* in that no recognizable cuticle is secreted by the serosal cells. The egg turns colour from yellow to dark brown soon after laying. There is, however, little morphological change in the chorion before and after the formation of the serosa. This morphological observation may be related to the fact that mercuric chloride is effective in breaking diapause before as well as after the formation of the serosa (KURIHARA and ANDO, 1969).

The difference in the effective time of action of chemicals between the eggs of *Atrachya* and *Melanoplus* may therefore be attributed to their different barriers for water absorption. One of the possible factors involved in diapause may be in the chorion in the former and in the serosal cuticle or wax layer in the latter.

This assumption may also be supported by the following fact. In crossings between non-diapausing and diapausing strains the diapause character of the egg is always the same as that of the mother (ANDO and MIYA, 1968). In several species of *Orthoptera*, however, the non-diapausing trait of the egg proved to be dominant in reverse crosses between non-diapausing and diapausing strains or species (BIGELOW and COCHAUX, 1962; HOGAN, 1965; MASAKI and OHMACHI, 1967). The chorion is secreted by the follicular epithelial cells of the ovary, while the cuticle is formed by the serosal cells which originate from the zygote nucleus. The diapause character, consequently, may be controlled maternally if the cause of diapause is in the chorion. On the other hand, it may be affected by either parent and the developmental physiology of the progeny may be determined by the dominant-recessive relations of the parent genes, if the cause of diapause is in the serosal cuticle or embryo.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Prof. K. MIYA and Mr. M. KURIHARA for their invaluable advice and encouragement during this work. Thanks are also due to Prof. S. MASAKI and Prof. T. O. BROWNING of the University of Adelaide for their critical reading of this manuscript.

REFERENCES

- ANDO, Y. and K. MIYA (1968) Diapause character in the false melon beetle, *Atrachya menetriesi* FALDERMANN, produced by crossing between diapause and non-diapause strains. *J. Fac. Agr. Iwate Univ.* **9** : 87-96. (in Japanese with English résumé).
- BIGELOW, R. S. and P. S. A. COCHAUX (1962) Intersterility and diapause differences between geographical populations of *Teleogryllus commodus* (WALKER) (Orthoptera: Gryllidae). *Aust. J. Zool.* **10** : 360-366.
- BODINE, J. H. (1932) Hibernation and diapause in certain Orthoptera. III. Diapause—a theory of its mechanism. *Physiol. Zool.* **5** : 549-554.
- BROWNING, T. O. (1969) The permeability of the shell of the egg of *Teleogryllus commodus* measured with aid of tritiated water. *J. Exp. Biol.* **51** : 397-405.
- FUKUDA, S. (1951) Factors determining the production of the non-diapause eggs in the silkworm. *Proc. Japan Acad.* **27** : 582-586.
- HASEGAWA, K. (1952) Studies on the voltinism in the silkworm, *Bombyx mori* L., with special reference to the organs concerning determination of voltinism. *J. Fac. Agr. Tottori Univ.* **1** : 83-124.
- HINTON, H. E. (1953) The initiation, maintenance, and rupture of diapause: a new theory. *Entomologist* **86** : 279-291.
- HOGAN, T. W. (1961) Termination of diapause in *Acheta* by urea. *Nature* **190** : 367-368.
- HOGAN, T. W. (1962) The absorption and subsequent breakdown of urea by diapausing eggs of *Acheta commodus* (WALK.) (Orthoptera: Gryllidae). *Aust. J. Biol. Sci.* **15** : 362-370.
- HOGAN, T. W. (1964) Further data on the effect of ammonia on the termination of diapause in eggs of *Teleogryllus commodus* (WALK.) (Orthoptera: Gryllidae). *Aust. J. Biol. Sci.* **17** : 752-757.
- HOGAN, T. W. (1965) Some diapause characteristics and interfertility of three geographic populations of *Teleogryllus commodus* (WALK.) (Orthoptera: Gryllidae). *Aust. J. Zool.* **13** : 455-459.
- KURIHARA, M. and Y. ANDO (1969) The effect of mercury compounds on breaking of diapause in the eggs of the false melon beetle, *Atrachya menetriesi* FALDERMANN (Coleoptera: Chrysomelidae). *Appl. Ent. Zool.* **4** : 149-151.
- LEES, A. D. (1955) *The physiology of diapause in arthropods*. Cambridge Univ. Press. London.
- MAKI, T. and Y. ANDO (1967) The influence of temperature on the egg diapause in the false melon beetle, *Atrachya menetriesi* FALDERMANN (Coleoptera: Chrysomelidae). *J. Fac. Agr. Iwate Univ.* **8** : 209-221.
- MASAKI, S. and F. OHMACHI (1967) Divergence of photoperiodic response and hybrid development in *Teleogryllus* (Orthoptera: Gryllidae). *Kontyû* **35** : 83-105.
- MIYA, K. (1965) The embryonic development of a chrysomelid beetle, *Atrachya menetriesi* FALDERMANN (Coleoptera) 1. The stages of development and changes of external form. *J. Fac. Agr. Iwate Univ.* **7** : 155-166.
- Official methods of analysis of A.O.A.C. (1960) 327-330.
- SLIFER, E. H. (1946) The effects of xylol and other solvents on diapause in the grasshopper egg; together with a possible explanation for the action of these agents. *J. Exp. Zool.* **102** : 333-356.
- UKITA, T., O. HOSHINO and K. TANZAWA (1963) Determination of mercury in urine, blood and hair of man in organic mercurial poisonings. *Eiseikagaku* **9** : 138-141. (in Japanese with English summary).
- WAY, M. J. (1960) The effects of freezing temperatures on the developing egg of *Leptohylemyia coarctata* FALL. (Diptera: Muscidae) with special reference to diapause development. *J. Insect Physiol.* **4** : 92-101.