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A NUCLEAR-POLYHEDROSIS VIRUS OF THE PAINTED APPLE MOTH (ORGYIA ANARTOIDES (WALKER))

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SUMMARY

A disease of the painted apple moth (*Orgyia anartoides* (Walk.)) caused by a nuclear-polyhedrosis virus was investigated. The virus is described on the basis of its morphology and pathology and the influence of larval age and size of virus dosage on incubation period. The polyhedra were distinguishable serologically from nuclear-polyhedra from *Heliothis armigera* Hubn., *H. punctigera* Wallengr., *Anthela varia* Walk, and *Pterolocera amplicornis* Walk. but not from *Orgyia australis* Walk. The viruses from *O. anartoides* and *O. australis* were reciprocally cross-infective, but the virus from *O. anartoides* was apparently not capable of infecting larvae of *H. punctigera* and *A. varia*.

I. INTRODUCTION

The painted apple moth (*Orgyia anartoides* (Walk.)) is a native insect which has extended its food range to include a number of cultivated plants. In the Stanthorpe area of south-eastern Queensland it is an intermittent pest responsible for partial defoliation of apple trees.

Cultures of this moth originating from Stanthorpe and maintained on apple leaves at the Indooroopilly Entomology Research Laboratory were found to have been destroyed by a previously unrecorded virus disease. Investigations on this disease, caused by a nuclear-polyhedrosis virus, are reported in this paper.

II. MATERIALS AND METHODS

Viruses.—The *O. anartoides* nuclear-polyhedrosis virus was found in larvae collected from apple (*Malus sylvestris* Mill.) at Stanthorpe by Dr. M. Bengston in February 1966 and January 1968.

Other nuclear-polyhedrosis viruses used in serological and cross-infectivity tests were as follows:—

ex *Heliothis punctigera* from larvae collected on lucerne (*Medicago sativa* L.) at Beaudesert, described by Teakle (1973);

ex *Heliothis armigera* from a larva in a laboratory culture, Brisbane;

ex *Orgyia australis* on apple at Holland Park, Brisbane;

ex *Anthela varia* on *Macadamia tetraphylla* L. Johnson at Sunnybank, Brisbane, described by Teakle (1969);

ex *Pterolocera amplicornis,* a small quantity of a dried preparation forwarded by Dr. T. D. C. Grace of the Division of Entomology, C.S.I.R.O., Canberra, A.C.T., and described by Day *et al.* (1953).

Larvae.—*O. anartoides* cultures were the progeny of larvae from apple trees at Stanthorpe in January and February 1969 forwarded by Dr. Bengston. These were reared on apple leaves when available. During the winter months leaves of geranium (*Pelargonium hortorum* Bailey) were used. Mass-rearing was done in gauze-covered cages containing branches held in jars of water. Individual rearing was done in 60 ml waxed paper cups containing a leaf with the petiole protruding through the base into a jar of water.

O. australis were collected on rose (*Rosa* sp.) at Virginia, Brisbane, and reared on geranium in the same way as *O. anartoides.*

Purification and counting of polyhedra.—Suspensions from triturated larvae were initially purified by filtration through two layers of cheese-cloth and several centrifugations for 1 min at 100 *g* to remove coarse material. Further centrifugations for 5 min at up to 1 000 *g* were done to obtain the purified suspension. The purity was largely gauged by eye but was checked periodically by phase contrast at 225X and 900X. The final suspension was white and contained a small amount of bacterial and uric acid contamination.

Polyhedra for infectivity tests were suspended in 0·2% 'Teepol' (wetting agent) to which 2 000 units each of penicillin and streptomycin were added to control bacterial infections.

Counts were done with a Hawksley-Helber counting chamber (Thoma ruling; depth 0·01 mm) using phase-contrast at a magnification of 225X. The suspensions were stored frozen at — 20°C until used.

Infection of larvae.—The larvae were infected by feeding leaves smeared with the virus dosage. Dosages, based on numbers of polyhedra, were prepared by appropriately diluting a stock suspension of known concentration in 0·2% Teepol and were applied using a Pasteur pipette calibrated for drop size.

Eggs were contaminated by dipping in a suspension of polyhedra in 0·2% Teepol containing a small quantity of fresh haemolymph as a sticking agent.

Histopathology.—Fifth instar (final or second last instar) *O. anartoides* larvae were used. Dosed larvae were sampled in pairs daily and examined after being killed with chloroform. One larva was fixed in Bouin-Dobosq-Brasil fixative for sectioning and staining by the method of Hamm (1966), while smears of blood and tissues from the other were examined immediately by phase contrast.

Electron microscopy.—A Siemens Elmiskop 1A electron microscope was used to determine the size and shape of polyhedra and virions within the polyhedra.

The size and shape of the polyhedra were determined from unstained preparations. For sectioning, the polyhedra were fixed in glutaraldehyde and embedded in Epon. Sections were cut on an LKB Ultratome and stained in uranyl acetate and lead acetate (Reynolds 1963). To reveal surface structures, and for measurement purposes, the liberated virions were negatively stained with 1 or 2% potassium phosphotungstate at pH 7·0.

Separation of virions from polyhedral protein.—The polyhedra were dissolved in 0·005 M Na_2CO_3 following the method of Bergold (1963). This gave complete degradation in 15 min. The virions were very fragile after removal of the outer membrane and modifications to the technique were made without success to obtain a greater proportion of them intact.

Serology.—The serological relationships between the polyhedral proteins of viruses were investigated using the Ouchterlony agar gel double diffusion technique (Crowle 1961, pp. 69-75).

(*a*) *Antigens.*—These were approximately saturated solutions of purified polyhedra from *O. anartoides, O. australis, H. punctigera* and *H. armigera* in 0·005 M Na_2CO_3–0·05 M NaCl and from *A. varia* and *P. amplicornis* in 0·015 M Na_2CO_3–0·05 M NaCl. Sodium azide to a final concentration of 0·5% was added to all antigens as a preservative (Koenig and Jankulowa 1968).

As the antigens consisted of approximately 95-97% polyhedral protein (Bergold 1963), that prepared from *O. anartoides* nuclear-polyhedra used for antiserum production was standardized using absorption at wavelengths 215 and 225 nm by the method for protein estimation of Waddell (1956). The polyhedral protein concentration obtained was approximately 2 mg/ml.

Control antigens were prepared by grinding 1 g of frozen healthy larvae in 10 ml of alkali of the corresponding concentration and clarified by centrifugation at 4 000 *g* for 5 min.

(*b*) *Antisera.*—Three guinea pigs each were used to prepare the antiserum to the *O. anartoides* virus. Each received an intraperitoneal injection of 3 mg antigen-protein followed by a second injection 1 day later of 1·5 mg antigen-protein in Freund's complete adjuvant. The guinea pigs were bled 3 weeks later and the sera were stored at — 20°C without added preservative.

(*c*) *Diluent.*—Veronal buffer pH 8·6, ionic strength 0·1, was used as a diluent to avoid possible precipitation of the polyhedral protein, which occurred at neutrality or a lower pH. A commercial veronal (barbitone) salts buffer (LKB-3276-VB) was used initially, and later a buffer consisting of 403 ml 0·025 M barbitone, 100 ml 0·5 M sodium barbitone and 100 ml 0·5 M sodium chloride per litre was substituted.

(*d*) *Agar gel diffusion tests.*—These were run in 9 cm diam. petri dishes in 12 ml 0·85% 'Difco' Special Agar-Noble containing 0·85% sodium chloride and 0·5% sodium azide as employed by Koenig and Jankulowa (1968), and buffered at pH 8·6 with veronal with an ionic strength of 0·1. The diameter of the reactant wells was 7 mm, the distance between the wells was 4 mm, and 0·025 ml volumes of the reagents were used. The concentration of the reagents was adjusted to give a single, well-defined precipitin line. The tests were done at 37°C overnight in a moist chamber.

Tests were carried out within several weeks of the preparation of the antigens as it was found that a specific component of the *O. anartoides* was lost during prolonged storage. This was indicated by the fact that the antiserum to a fresh preparation of this virus showed greater reactivity with some antigens prepared from other viruses than the *O. anartoides* antigen after storage.

(*e*) *Ring precipitin tests.*—Antigen, diluted 1 in 4 in pH 8·6 veronal buffer, was layered on to undiluted serum in 5 x 0·5 cm Durham tubes. The tubes were examined for precipitation at the interface at from 1 hr to 3 hr at room temperature and also after overnight refrigeration.

III. RESULTS

(a) Description of Virus

Polyhedra.—The polyhedra were irregular in shape and contained the virions embedded singly and at random within (Figure 1). The diameters of the polyhedra and sizes of the virions are given in Table 1.

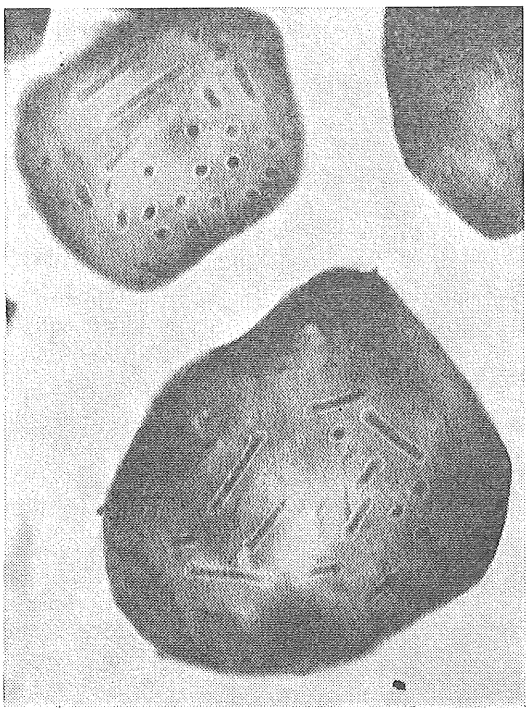

Fig. 1.—Sections of nuclear-polyhedra from *Orgyia anartoides,* showing the embedded, rod-shaped virions.

TABLE 1

DIAMETERS OF POLYHEDRA AND LENGTH AND BREADTH OF VIRIONS FROM A NUCLEAR-POLYHEDROSIS OF *ORGYIA ANARTOIDES*

Particle Measured	Number Measured	Dimension	Range (nm)	Mean ± S.E. (nm)
Polyhedra	281	Diameter ..	600–2 100	1 141 ± 10
Virions with outer membrane ..	100	Length	250– 490	330 ± 2·9
	100	Breadth ..	54– 90	70·6 ± 0·8
Virions without outer membrane	14	Length	275– 410	360 ± 10·4
	14	Breadth ..	45– 60	51 ± 1·2

The random arrangement of the virions within the polyhedral protein matrix was apparent in ultra-thin sections. The occluded virions did not disturb the lattice of polyhedral protein, which was a regular 90° arrangement of the molecules.

Difficulty in sectioning the polyhedra was experienced owing to their hardness, at least at their surface, which caused chipping of the glass knife. A narrow, dark staining band was present at or near the periphery of some sections of polyhedra, suggesting a limiting membrane. A collapsed-tent-like structure often enclosing virions was also frequently observed when polyhedra were degraded on the grid with dilute sodium carbonate or with phosphotungstate adjusted to pH 11 with 10% KOH. This appeared to be an alkali-resistant outer layer to the polyhedron and probably corresponds to the dark-staining band in sections.

Virions.—The degradation of polyhedra with dilute alkali brought about the release of virions enclosed within a double outer membrane. Continued treatment with alkali brought about degradation of the outer membrane, allowing the negative stain to penetrate along the length of the rod but not at the ends, resulting in the appearance of a cap at both ends. Rupture almost invariably occurred lengthwise, allowing the naked virion to be observed still partly enfolded in the membrane or in the free state. Occasionally virions twice the normal length were seen. When the outer membranes were shed, the virions tended to elongate as seen in Table 1, suggesting that the inner membrane is a flexible, continuous structure. Surface striations on the inner membrane at 45° to the axis were occasionally seen, possibly corresponding to an internal helical structure. A number of disc-like or short cylindrical forms 25–30 nm in diameter with a central hole approximately 20 nm across were associated with some of the virions, but their origin was not apparent.

(b) Serological Relationships

An attempt was made to distinguish between this virus and nuclear-polyhedrosis viruses from *O. australis, H. punctigera, H. armigera, A. varia* and *P. amplicornis* on the basis of the antigenic characteristics of the polyhedra. The antiserum preparation giving the strongest reaction with the corresponding *O. anartoides* virus antigen in a preliminary ring precipitin test was used in agar gel diffusion tests.

No reactions were given by antigens prepared from normal host insects, or when non-immune guinea pig serum was used.

Each of the viral antigens reacted strongly with the antiserum to the *O. anartoides* virus, and when comparisons of the antigens was made patterns of 'identity' (fusion) or 'partial identity' (partial intersection) (Crowle 1961, p. 70) were obtained. The reactions given in Table 2 indicate that the nuclear-polyhedra from *O. anartoides* and *O. australis* have a similar, specific antigenic component not possessed by those from the other insects.

(c) Pathology

Symptoms and signs.—The disease was normal with respect to symptoms and signs (Aizawa 1963). Infected larvae usually showed no symptoms or signs until 1–3 days before death, when they became sluggish and irritable, ceased feeding, and a creamy pallor of the non-pigmented areas was noticeable. The larvae finally became moribund and swollen, with pronounced segmentation. At death they had a pasty appearance of the non-pigmented areas and often died from

TABLE 2

AGAR GEL DIFFUSION REACTIONS GIVEN BY PAIRS OF ANTIGENS PREPARED FROM NUCLEAR-POLYHEDRA FROM *ORGYIA ANARTOIDES*, *ORGYIA AUSTRALIS*, *HELIOTHIS ARMIGERA*, *HELIOTHIS PUNCTIGERA*, *ANTHELA VARIA* AND *PTEROLOCERA AMPLICORNIS* WITH ANTISERUM TO *ORGYIA ANARTOIDES* NUCLEAR-POLYHEDRA PREPARED IN GUINEA PIG

Antiserum	Antigen Pair										
	O	O	O	O	O	Oau	Oau	Oau	H	H	Ha
	Oau	H	Ha	A	P	H	Ha	A	Ha	A	A
Orgyia anartoides nuclear-polyhedrosis virus	I	Pi (O)	Pi (O)	Pi (O)	Pi (O)	Pi (Oau)	Pi (Oau)	Pi (Oau)	I	I	I

Pi —reaction of partial identity; virus showing additional specific antigenic group is given in brackets.

I —reaction of identity.

O —*Orgyia anartoides* nuclear-polyhedra.

Oau—*Orgyia australis* nuclear-polyhedra.

Ha —*Heliothis armigera* nuclear-polyhedra.

H —*Heliothis punctigera* nuclear-polyhedra.

A —*Anthela varia* nuclear-polyhedra.

P —*Pterolocera amplicornis* nuclear-polyhedra.

a high point in the rearing container with the anterior part or the whole of the body hanging vertically (Figure 2). The bodies were soft and the skin fragile, and when handled a creamy exudate containing large numbers of polyhedra leaked out. The cadavers darkened rapidly and were quickly invaded by bacteria.

Histopathology.—The infection was first noted at 5 days as a general hypertrophy of nuclei and cells of the fat body, hypodermis and tracheal matrix. Polyhedra were present in a low percentage of the cells. At 7–9 days the larvae were in advanced stages of infection. Most of the blood cells and the cells of the fat body, hypodermis and tracheal matrix were packed with polyhedra (Figures 3, 4), and infection of the muscular sheath and lining of the ventral nerve cord was apparent. An unusual feature was the infection of some cells of the silk gland. A number of the blood cells contained small numbers of polyhedra in the cytoplasm, apparently phagocytosed. Death occurred 6–10 days after dosing.

The infection of the silk glands is relatively uncommon, although Aruga, Fukuda and Yoshitake (1963) observed polyhedra in nuclei of the silk gland of nearly all infected silkworm larvae of the later (third to fifth) instars, but the silk glands of earlier instar larvae had no polyhedra. Benz (1963) observed that most glandular cells which have a high protein metabolism prevent, or at least slow down, the development of these viruses. The fact that the silk glands are, as a rule, attacked only towards the final stages of the disease is possibly because the general metabolism of the insect (and with it the metabolism of the glandular cells) is disturbed.

Infection in final instar larvae.—It was apparent that the infection of final instar larvae tended to prolong their larval life (Table 3).

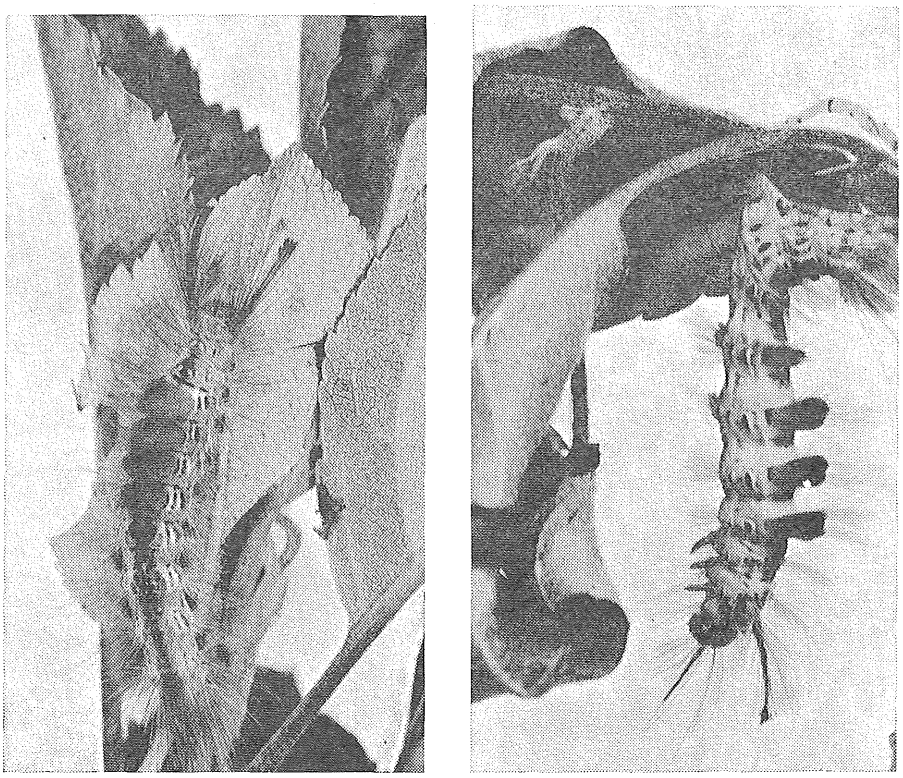

Fig. 2.—Left, healthy *Orgyia anartoides* larva; right, larva killed by nuclear-polyhedrosis.

TABLE 3

COMPARISON OF THE INCUBATION PERIODS OF NUCLEAR-POLYHEDROSIS IN FINAL INSTAR LARVAE OF *ORGYIA ANARTOIDES* WITH TIMES TO PUPATION OF THE UNTREATED LARVAE

Normal Larvae			Infected Larvae		
Number of larvae	Number of Days to Pupation		Number of Larvae	Incubation Periods (days)	
	Range	Mean		Range	Mean
7	7–11	8·6	18	7–12	10·0

The differences between incubation periods and times to pupation (i.e. termination of larval life in each case) were significant ($P < 0{\cdot}02$).

Final instar *O. anartoides* larvae with nuclear-polyhedrosis rarely pupated successfully and then succumbed to the disease in the pupal stage. Two male larvae which pupated 2 and 3 days respectively after dosing became yellow and soft and failed to emerge as adults. Polyhedra were present in smears of the body contents.

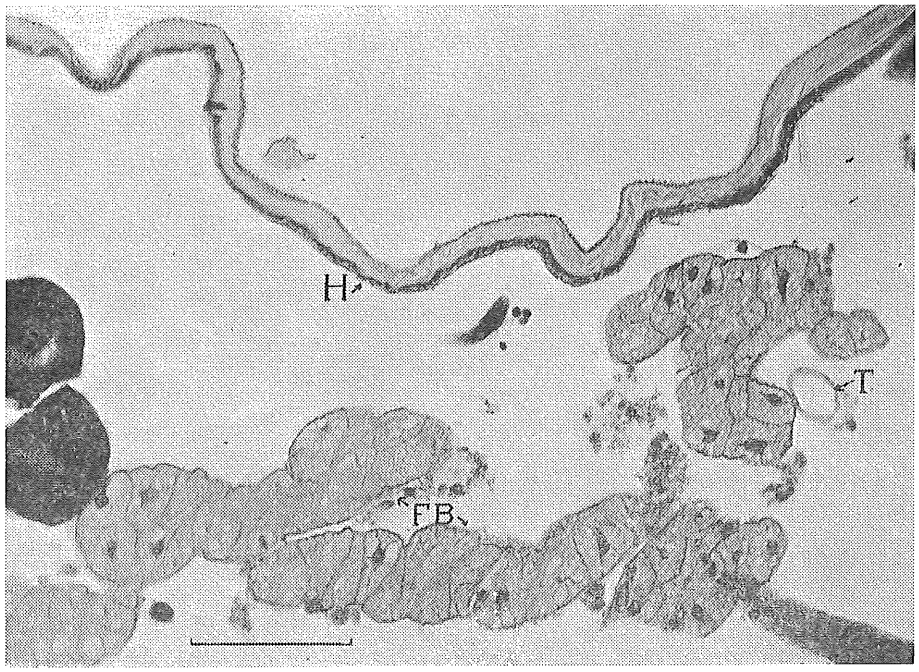

Fig. 3.—Transverse section of normal *Orgyia anartoides* larva. H, hypodermis; FB, fat body; T, tracheal matrix. Line represents 0·1 mm.

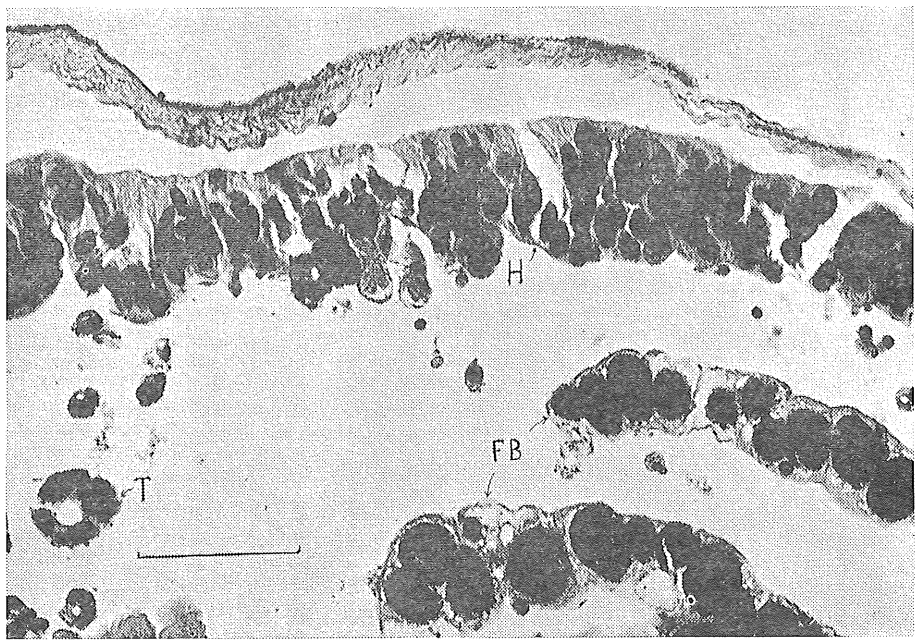

Fig. 4.—Transverse section of *Orgyia anartoides* larva 8 days after dosing with nuclear-polyhedrosis virus, showing massive hypertrophy of nuclei and cells. The hypodermis has undergone an approximately tenfold increase in thickness. H, hypodermis; FB, fat body; T, tracheal matrix. Line represents 0·1 mm.

TABLE 4

INCUBATION PERIODS OF NUCLEAR-POLYHEDROSIS OF *ORGYIA ANARTOIDES* DETERMINED FOR EACH INSTAR

Insect Stage at Treatment	Dosage (polyhedra)	Incubation Period (days)		Number Dosed	Number Dead		Number Moulted	Number of Days Infected Before Moulting
		Range	Mean		Polyhedrosis	Other Causes		
Egg (surface-contaminated)	$1{\cdot}5 \times 10^9$	1–10	6·7	19	12	7	6	4–6
Larva 1st instar (confined individually)	4×10^7	5–9	6·6	13	13	0	1	4
1st instar (batches of 5) ..	2×10^8	5–8	6·7	19	16	3	3	5
2nd instar (confined individually)	4×10^7	6–10	7·0	20	20	0	4	4
3rd instar (confined individually)	4×10^7	7–9	7·2	19	19	0	3	4–5
4th instar (confined individually)	4×10^7	6–10	7·4	14	14	0	9	2–3
5th instar (confined individually)	4×10^7	5–11	9·0	18	17	1	7	3–4
6th instar (confined individually)	4×10^7	7–12	9·6	26	21	5	—	—

(d) Infectivity Tests

In order to further understand the interaction between host and pathogen, infectivity tests were set up to determine the effects of larval age and virus dosage on incubation periods and larval mortality. Cross-infectivity tests were also done.

Effect of larval age on incubation periods and mortality.—Data relating to the incubation period of the disease and mortality following dosing of larvae of all six instars are given in Table 4. As first instar larvae tended to be gregarious, they were confined both individually and in batches of five to determine whether any change in susceptibility resulted from isolation.

Little difference was recorded between mean incubation periods of the first instar larvae confined individually or in batches, or, in fact, between the mean incubation periods of the larvae of the first four instars. A more marked increase in incubation periods was noted for larvae of the fifth instar, which included final instar male larvae, and larvae of the sixth instar, which were all final instar female larvae.

All larvae succumbed to infection irrespective of instar. However, on the basis of the incubation periods, it appeared that larvae of the earlier instars were more susceptible to the nuclear-polyhedrosis than those of the later, particularly fifth and sixth, instars.

Effect of virus dosage on incubation periods and mortality.—Dosages ranging from 10^2 to 10^7 polyhedra were fed to sixth instar larvae, all final instar female larvae. The results are given in Table 5.

TABLE 5

EFFECT OF VIRUS DOSAGE ON INCUBATION PERIOD OF NUCLEAR-POLYHEDROSIS AND MORTALITY IN FINAL INSTAR *ORGYIA ANARTOIDES* LARVAE

Dosage (polyhedra)	Number of Larvae Dosed	Number Dead		Number Pupated	Incubation Period (days)		Mortality (%)
		Polyhedrosis	Other Causes		Range	Mean	
0 (Control)	5	0	0	5	—	—	0
10^2	7	2	0	5	12–14	13	30
10^3	8	0	0	8	—	—	0
10^4	5	2	0	3	13	13	40
10^5	5	4	0	1	12	12	80
10^6	7	7	0	0	11–14	12	100
10^7	5	5	0	1*	10–13	11	100

* Dead of polyhedrosis at 18 days.

The incubation periods were similar for dosages over this range. As mortality occurred with as few as 10^2 polyhedra and survival of larvae occurred with dosages of 10^5 polyhedra, the thresholds for infection of the individual larvae apparently differed considerably. However, when the threshold had been exceeded the time-course of the infection was generally similar.

Cross-infectivity tests.—The reciprocal cross-infectivity of the *O. anartoides* virus and the viruses from *O. australis, H. punctigera* and *A. varia* was investigated. These indicated that the viruses from *O. anartoides* and *O. australis* were reciprocally cross-infective, but that *O. anartoides* was not susceptible to infection by the viruses from *H. punctigera* and *A. varia.* One of 10 *A. varia* larvae dosed with the *O. anartoides* virus died of polyhedrosis, but subsequent tests involving dosages of 10^6 polyhedra from this particular insect showed it to be non-infective for *O. anartoides* and infective for *A. varia,* and this virus apparently resulted from pretest infection. In two tests in which *H. punctigera* larvae were dosed with the *O. anartoides* virus, none of 11 larvae died of polyhedrosis in one, but in the other, 8 out of 9 dosed with another batch of virus died. Extracts of individual larvae were made as described by Teakle (1973) to determine the serological identity of the virus present by agar gel diffusion. Two of the extracts gave well-defined precipitin lines which indicated that the virus present was of the *Heliothis* and not the *Orgyia* type. The antigenic nature of the polyhedral protein is presumably determined by the viral genome and not the host, and these results suggest that the infections resulted from accidental contamination with *Heliothis* virus, or activation of a latent virus.

IV. DISCUSSION

The reciprocal cross-infectivity and identical serological reactions given by the viruses from *O. anartoides* and *O. australis* suggest that they might be, in fact, the same virus. The ecological significance of the possible mutual exchange of viruses between these two insects is not known. While these species occur on a similar range of host plants, their geographical distribution varies, and *O. anartoides* is not known to occur in the Brisbane area (I. F. B. Common, private communication 1969) where the original virus-infected *O. australis* larvae were collected. Hence the significance of this fact cannot be assessed without the knowledge of alternative host insects and the vectors of the viruses.

The nuclear-polyhedrosis virus studied in the current work is a virulent pathogen. The habit of *O. anartoides* of depositing eggs in clumps and the gregarious tendency displayed by early instar larvae favours early cross-transmission of the virus. Subsequent cross-transmission is favoured by the relative lack of mobility of the female adult, which is wingless; this tends to restrict the distribution of this insect to individual trees. It is apparent that the presence of the virus must be considered in any future work on the ecology of *O. anartoides.*

The broad spectrum pesticides currently required for the control of major pest species in apple orchards in Queensland also control *O. anartoides* and it is unlikely that the virus will find application for the control of *O. anartoides* for the present. However, the virus has potential should pest management programmes currently being developed for apple orchards become widely adopted.

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