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Stressor Effects of High Relative Humidity and a High Moisture Diet on appearance of Disease from Nuclear Polyhedrosis Virus in Larvae of Porthetria Dispar (L.)

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STRESSOR EFFECTS OF HIGH RELATIVE HUMIDITY AND A HIGH MOISTURE DIET ON APPEARANCE OF DISEASE FROM NUCLEAR POLYHEDROSIS VIRUS IN LARVAE OF PORTHETRIA DISPAR (L.)

PAULINE A. THOMAS

1977

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May 2, 1977
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STRESSOR EFFECTS OF HIGH RELATIVE HUMIDITY

AND A HIGH MOISTURE DIET

ON APPEARANCE OF DISEASE FROM NUCLEAR POLYHEDROSIS VIRUS

IN LARVAE OF PORTHETRIA DISPAR (L.)

Pauline A. Thomas

B.S. Yale University 1973

A Thesis submitted to the Faculty

of the Department of Epidemiology and Public Health

of the Yale University School of Medicine

in partial fulfillment of the requirements for the

Degree of Doctor of Medicine

1977

To my sister Lisa

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ACKNOWLEDGEMENTS

ABSTRACT

High relative humidity (R.H.) and a high moisture diet were tested as stressors in the incidence of nuclear polyhedrosis virus (NPV)disease of larvae of <u>Porthetria dispar</u> (L.). Relative humidity was shown to have no effect on the appearance of NPV disease in larvae reared in the laboratory. However, a high mortality from other causes was sustained by a group of larvae subjected to both high R.H. and a high moisture diet. The absence of NPV disease during the first larval instar period confirmed earlier findings that effective egg sanitization virtually eliminates NPV disease. This supports current disfavor with the theory of transovarial transmission of <u>P. dispar</u> NPV. One case of NPV disease was found in a late instar larva. This may be attributed to incomplete sanitization of its egg, or to later contamination.

The problems encountered here in studying latent viral disease, and in pinpointing the roles of certain environmental conditions as stressors, parallel similar obstacles to the study of stressor action and latency in human disease.

The nature of insect nuclear polyhedrosis virus is discussed, as well as the potential effects on public and environmental health of its use as a viral insecticide.

LARVAL INSTARS (approx. sizes)   EGG

5th   4th   3rd   2nd   1st

PUPA (approx. size)

ADULT FEMALE
(approx. 1.5 x actual size)

ADULT MALE
(approx. 1.5 x actual size)
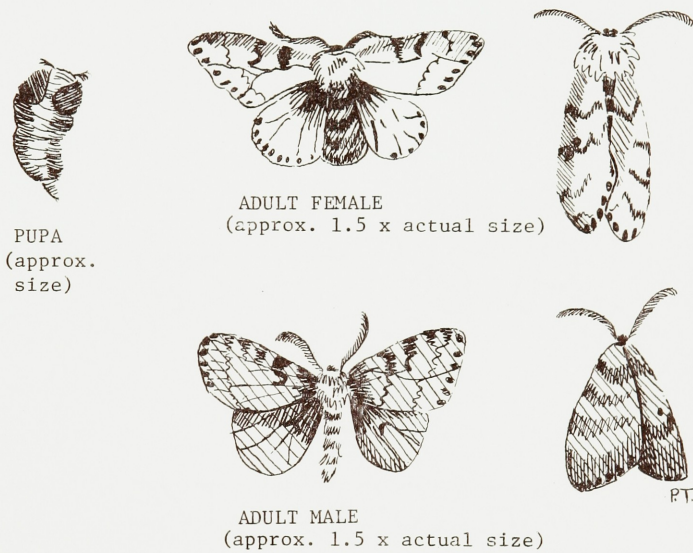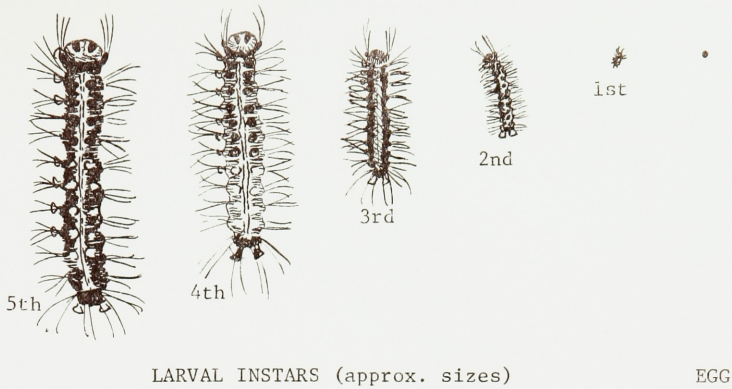
FIGURE IV.   General appearance of developmental stages of
the gypsy moth (Porthetria dispar).

INTRODUCTION

Environmental Factors in Disease.

Physicians, general biological researchers, and others dealing
with disease in man or in other animals began long ago to puzzle
over a common observation that two similar individuals of two
populations having a similar amount of exposure to a pathogen will
often react differently. One may be mortally stricken, and the other
remain quite healthy; or both may evince disease, but show very
different signs of it. With our growing knowledge of immunity, we
have acquired some understanding of factors responsible for this
puzzle in man and other mammals. Immunity is one factor playing a
role in the host-disease relationship. In humans, "memory" of the
T lymphocytes might enable a person at a second exposure to fight
off an infection, or in the case of allergy may make a second contact
with a foreign substance more dangerous. An inborn or acquired
defect in the ability to form antibodies, or a momentary overpowering
of available antibodies with excess antigen, may cause one person
to succumb to an invading pathogen which another may ward off with
ease.

Another factor accounting for different responses to pathogens
may be found in the specific environment inhabited by the individual.
Environmental agents may disturb physiological function and interfere
with host defenses; or they may disturb structure, and in some way

facilitate entry of a pathogenic organism into host tissue. For

example, after long years of cigarette smoking, lung structure may

be damaged, resulting in larger amounts of sputum production. The

conditions created in the lung by smoking are then ideal for various

pathogens -- e.g. Hemophilus influenza, a common pathogen of patients

with chronic bronchitis -- and greatly inhibit clearance of pathogens

from hosts' lungs. A nonsmoker inhaling the same number of H.

influenza organisms may experience an abortive infection.

The environmental agents (usually much more subtle agents than

cigarette smoke) which affect the host's ability to cope may be

called "stresses". The same term has been used to describe the

host's responses to such agents. For greater clarity the latter

definition will be used here, and "stressor" will be used to apply

to the agents themselves.

One scholar in the field of human stress, Selye (1976), notes

that a distinction must be made between specific consequences of the

action of a stressor on an organism and nonspecific responses that

may be evoked in the organism by any stressor. He defines as a

stressor any endogenous or exogenous agent which forces the organism

to adapt to a change in its environment. In man this stressor may

be anything from a physical or chemical injury (e.g. poisoning) to

emotional upset, or a change in social structure. The human organism,

at least, experiences the same biochemical hormonal responses to

these various stressors.

The specific effect of a stressor in human disease is less

easily determined. An environmental agent may have an effect on

an organism's ability to survive and remain healthy, and yet not
have an enhancing effect on the development of a particular disease.
Smoking may be just such an environmental agent. But even in cases
where particular diseases may seem to follow particular stressor
action, the real relationship existing between the two may be very
subtle. A major problem in the study of stressors in man is that
physiological, sociological, and psychological effects overlap.
For example, a sociological problem may lead to psychological
distress which in turn causes general and specific physiological
disturbances, which may manifest themselves as ulcers, colitis,
or a behavioral disturbance. Which entity is the immediate "stressor"
is difficult to point out (Lazarus, 1971). At least in the gastro-
intestinal disturbances just noted, and in pathology stemming from
the habit of smoking, it is accepted that there is a relationship
between the disease and the environment. However, defining the exact
environmental stressors which affect the behavior of a particular
disease in an individual, and the exact site of action of that
stressor is difficult.

An area where such a stressor effect on response to a pathogen
is strongly suspected is in infection with Herpes simplex virus. In
those infected with this virus, a first attack of acute stomatitis
often occurs in childhood; and then throughout life there are
recurrences, seemingly stimulated by triggering influences, e.g. ultra-
violet light, exposure, fever, menstruation, nerve injury, or emotional
disturbances (Fenner et al., 1974). The effects of these on the
host are not thought to be stresses which aid in invasion by the

virus, but they do seem to activate a pathogen already present. Various mechanisms whereby the herpes virus may maintain itself in the cranial nerve ganglia have been proposed, and are reviewed by Fenner, et al., (1974). The effects of other stressors are less widely accepted and even more poorly understood. Carcinogens may be stressors in viral disease, where certain cancers have viral etiologies. However, they will not be understood until the diseases themselves are better explained.

Whether one is examining herpes or cancer, or an infectious viral disease like polio (an environmental study on different manifestations of disease by identical strains of polio virus was done by Evans, 1960), environmental effects must be understood in order to understand individual susceptibilities. A Russian pathologist, Pavlovskii, (noted in Audy, 1958) suggests that there is a habitat of disease which includes the microenvironment of the tissue within the organism infected by the disease agent, but which also includes the environment in which the host organism itself lives. Habitat of disease may refer not only to the individual case, but to the surrounding environment in which the disease is found. An obvious example in humans is an arthropod borne disease which relies for continuing dispersal on invertebrates which could not survive cold winters. A less obvious example is the puzzling epidemiology of multiple sclerosis, which seems to occur predominently in cooler latitudes, and in females.

When a disease occurs only in one area it is often difficult to determine whether a particular pathogen can only survive in that

area, or whether there is some element, a stressor, existing in that area, which causes susceptibility to the pathogen. Certainly such problems have public implications, and may be sorted out, over time. It is hoped that new methods may be devised to make the sorting more possible, and then more efficient and rapid.

Non-human Disease Playing a Role in Man's Environment

Non-human disease is one factor which may influence man's environment. This is not introduced here because of any role it may have as a direct stressor influencing the course of any particular disease. It is introduced first because it may cause great general stress to humans. Crop damage from a fungus may cause famine; forest damage from similar causes may engender economic distress; illness among livestock can take a financial toll, and also in some cases serve as a disease threat to humans. Insect disease, the subject of the study to be presented in this paper, may also influence man's environment. Disease in pest populations may erratically or steadily alleviate the difficulties caused by the pest. That of helpful insects obviously will lessen benefits derived from those insects.

Non-human disease is also of interest in that it can aid in our understanding of human afflictions. Of common knowledge are contributions gained from study of the diseases of other mammals -- guinea pigs, rats, cats, dogs, sheep. Less known and less used, but of historical importance and of importance in continuing research, are diseases of organisms less closely related to the human one.

Perhaps the most unusual case is the role of plant disease in the better understanding of disease in general. For example, virologists have used tobacco-mosaic virus as a research tool. Another little known example is that of insects, which have provided some major clues about disease mechanisms. The first experiment which demonstrated a microorganism to be the cause of a disease was in an insect, the silkworm (Bombyx mori). The microorganism was a fungus and the experiment was performed by Agostino Bassi in 1834 (Steinhaus, 1960b). Louis Pasteur's first work was with two other diseases of the silkworm (Ibid.) and other scholars of the nineteenth and twentieth centuries have engaged in further work on insects. In this century, the greatest effort is directed toward pathogens of humans and other animals, as carried by insects, which may not cause disease in the insects themselves. But study continues in the field of insect pathology itself. Some applications are obvious. Diseases of the carriers of human pathogens may alter the behavior of the carriers and thus change the incidence of distribution of human disease. It well behooves man to learn more of the pathology of useful creatures like the silkworm, or honeybees, in an effort to maximize benefits from them.

Another major use of work in insect pathology is in the control of insects, both disease carriers and agricultural pests. Pest control has a long history, and methods used have been of various degrees of usefulness, effectiveness, and safety. Until the end of the Middle Ages insect control was limited to mystical and physical varieties. Then infusions of tobacco used to fight lacewings on

pear trees, and, in the 19th century, use of a chemical extract of chrysanthemum, Pyrethrum, marked the beginning of chemical warfare against insects (Stoler, 1976). In the 1940's DDT (Chlorophenothane), a host of other chlorine derivatives, and phosphorus based compounds were found effective and were begun to be used for their powerful insecticidal capabilities. From 1940 to 1975 there was a 10% increase in crop yield where the new insecticides were used. But as early as the late forties researchers began finding traces of DDT in higher life forms (Stoler, 1976), and Rachel Carson's Silent Spring in 1962 dispersed among a wide audience some understanding and fear of where the wide use of chemical insecticides could lead. While DDT has been banned since 1972, organophosphorus compounds, some arsenic containing compounds, as well as other chemicals, continue to be used. All can, in various ways, be toxic to human beings. It is also being found that many insects with their short generation times, have a capacity for becoming resistant to chemical poisons. In the search for control agents which are noxious for the insect alone, a more sophisticated approach to insect pathology can play a role (i.e. an approach involving more than just the knowledge of what is poisonous to the insect). Control of an insect with a disease agent falls under the heading of biological control. Other forms of control also belong under this heading. One is the use of imported predators and parasites, begun in the 19th century. Another is the use of biological tricks played on the insects, including the release of sterile males, and the luring of insects into traps by the use of pheromones (Stoler, 1976).

Use of juvenile hormone can be likened to use of a poison specific for the insect - a clever idea, which unfortunately has one of the same drawbacks as the chemical insecticides: in the laboratory, after fifteen generations, houseflies and mosquitoes developed resistance (Ibid.). These biological control methods are derived from what has been learned of insect physiology rather than insect pathology. The latter finds application specifically in the area of microbial control. This method has not yet had wide application, but at least three microorganisms have been approved by the Environmental Protection Agency for use in the United States: One bacterium, Bacillus thuringiensis, has been used against various insect larvae. In addition, two viral insecticides have been approved by the EPA: one in the past year for use against the spruce budworm (Environment News, 1976); and another used in recent years against the cotton bollworm (Heliothis Zea) (Greer, et al., 1971).

The introduction of a pathogen into the environment in large quantities may be a very risky procedure, and together, the Food and Drug Administration of the U.S. D.H.E.W., and the Pesticide Regulation Division of the U.S.D.A., have elaborated requirements for registering such agents for use. These provide for guarantees of standardization, uniformity, and purity of the product; efficacy as proven by field tests; safety for vertebrates and beneficial insects; and a level of tolerance or exemption from tolerance covering food and feed crops which may be contaminated (Heimpel, 1967). When the fulfillment of these requirements is guaranteed microbial control may

be a useful adjunct (or a replacement for) methods already available
for pest control. However, continued monitoring must also be initiat-
ed. There is among viruses and bacteria (as is well known from the
past century of active research) a great ability for mutation, and
even if not a present danger this ability to change might pose a
potential future threat to other insects in the environment, as well
as to warm-blooded animals. As already noted the insects themselves
have a remarkable ability over a few generations to develop resistance
to many agents. Most of the pathogens proposed for use are natural
pathogens of the insects involved, and presently exist in low or
varying levels in the field populations. Introduction of larger
numbers of pathogens could upset the habitat of the pathogen -- e.g.
by killing off individuals which would otherwise harbor the pathogen
at low levels -- allowing it to be passed on to the next generation
without inevitably causing mortality. With the artificially imposed
larger dose, animals which failed to succumb might have special
resistance, and obviously the survivors would be the ones to establish
the succeeding generation of insects. Such a process, an upset in
the natural balance between pathogen and host, could lead to the
establishment of a whole population having increased resistance to
the pathogen. Any risks to the health of other species or to man, or
risks involved whenever the balance of predator-prey relationships
is shifted (e.g. overproliferation of another insect which may have
been a prey of the insect being regulated by microbial control) would
hardly be justified if the evolutionary changes in pathogen or target

insect occurred too quickly for successful, prolonged control of the pest.

The Nuclear Polyhedrosis Viruses

The study undertaken here involves the gypsy moth, Porthetria dispar (Linnaeus) and a virus which exists in most North American populations of P. dispar, and which has been proposed as a microbial control agent against the moth. There are at least three types of viruses which are pathogenic for insects and which produce inclusion bodies: granulosis viruses (GV), cytoplasmic polyhedrosis viruses (CPV), and nuclear polyhedrosis viruses (NPV) (Mazzone et al, 1976). All three are being studied for their potential in microbial control, but in some ways the last seems most promising. NPV's are usually host specific, while CPV's and GV's are more often infective for more than one insect species. The fact that NPV's can be mass produced (by harvesting them from infected larvae reared in the laboratory) and that they are in nature powerful pathogens to their host insects have contributed to their appeal as candidates for microbial control agents. The two viruses mentioned earlier as already approved by the EPA for field use are NPV's.

Physical details of the P. dispar NPV will be covered presently, but first the general regulation and development of NPV's will be discussed. These viruses are subject to the same basic regulations enumerated earlier for application to pathogenic biological control

agents. Most important, perhaps, are the studies aimed at insuring their safety for other organisms: effects of intravenous and intraperitoneal injections of rodents, oral toxicity of rats, oral allergenicity, carcinogenicity, mammalian tissue culture studies, human exposure by feeding, data on inhalation and dermal exposure to the polyhedra of the virus being tested, and the effect on honeybees (Apis mellifora, L.) (Heimpel, 1967). Some of these tests are done only with the polyhedra (the crystallike inclusion body, enclosing a number of polyhedrosis particles -- rod shaped virions, in NPV) (Mazzone et al., 1976) while others are performed using the virions themselves, free of the polyhedral protein.

A great deal of thought has been given to the hazards of application of virus in the environment, as may be surmised from the necessary tests described above to which the virus must first be subjected. However, Heimpel (1976) points out that the polyhedrosis viruses proposed as control agents would not be new additions to our environment. They are natural pathogens, to which many animals have already been exposed. Raw green leafy vegetables are particularly likely to have a few polyhedral bodies left on them by infected larvae. Work has already been done to determine whether NPV's have infected woodland mammals or laboratory workers who have come into contact with large number of infected insects. In neutralization tests on serum of laboratory workers exposed for more than ten years to silkworm NPV, no evidence of NPV antibodies was

found (Aizawa, 1954). Only one virus similar to an insect poly-hedral virus has been described in an animal that is food for man: an occluded virus noted in shrimp (Couch, 1974).

In addition to serological studies of laboratory workers, a survey of personnel in known laboratories has been done to determine whether the workers there have been subject to any unusual symptoms of infections. As of 1967, there were no unusual findings of any sort although some workers had received what must be described as gross exposure (Heimpel, 1967). In addition, there has been no evidence of wildlife disease or increased mortality in areas where the virus has fluorished (Ibid.).

The above evidence has been taken as indirect proof that the insect NPV's are not infective outside of the order Insecta. It has usually been noted that among insects NPV's are specific for individual host species. It is hoped that the required experiments to test possible infectivity to honeybees may be done carefully, and perhaps extended to other useful insects, in order to increase the evidence that the viruses are not only order-specific, but truly species specific.

The suggestion has been made by Shope (in discussing a paper presented by Mazzone, 1975) that constant monitoring must be continued with these agents, to insure against hybridization with existing natural viruses. It must also be insured that independent mutation will not occur, enabling the virus to infect animals other than insects. There is little evidence to date that mammalian cells can

support the growth of NPV, or the production of polyhedral bodies.
Himeno and his coworkers (1967) after injecting infective DNA of the
NPV of Bombyx mori (the silkworm) into a culture of human amniotic
cells, reported the production of polyhedral bodies which were sub-
sequently shown to be infective for the silkworm. However, the
number of silkworms infected with the suspect polyhedra was small,
and statistical significance of their relative infection rate is
difficult to evaluate. Knudson (1975) was unable to duplicate the
findings in a similar experiment. Infectious DNA was injected into
human amniotic cell culture, as well as into live hamsters intra-
peritoneally, intracerebrally and subcutaneously. No cytopathic
effects were observed, and no polyhedra appeared, although the
infectivity of the DNA used was verified by injecting it into larvae,
in which it produced a substantial rate of infection.

Viruses can undergo spontaneous mutation and it is thought
that some viruses have set rates for this. Also, chemical and
physical mutagens have been described for some groups of viruses
(Fenner et al., 1974). Tinsley, et al., (1974) note that "host
specificity is neither a fundamental nor a stable characteristic" of
viruses. However, Heimpel suggests (1967) that mutation of an insect
NPV to a mammalian or a human pathogen is a needless worry. Without
the ability to invade a test organism, a virus is unlikely to develop
pathogenicity for that organism; that is, mutants pathogenic for
that organism will not be selected for. NPV's when ingested are

dissolved in gastric acid and the virions, sensitive to acid, are also destroyed (Bergold, 1963; Heimpel, 1967). Thus viral invasion is usually not possible via the oral route, the principle way most warm blooded animals may come in contact with polyhedra. In persons with achlorydria the case may be somewhat different. Alkaline pancreatic secretions (pH 7.5 to 8.5) (Harrison's, 1974) may cause dissolution of polyhedra without destruction of virions. It might be advisable to test this route of possible viral invasion in an animal model.

If the polyhedral protein remains intact, infection may be impossible. It has been shown by injection of NPV into hemolymph of insects, that they are not infective when the polyhedral protein remains undissolved. The other possible routes of mammalian invasion, by inhalation or transdermally, will probably not allow release of virions, as those routes would not cause exposure of the polyhedral proteins to extremes of pH, or to proteolytic enzymes. However, it is not proven that viral invasion could not occur by one of those routes. The proposed method of application of the virus to vegetation involves an aerosolized form, the spraying of which would make it more likely for an individual to inhale the virus than in the natural setting where the virus is unlikely to blow around. Many polyhedra are of a size which makes then well able to penetrate into lung alveoli. They may be 0.3 to 15 microns in diameter, depending on species (Heimpel, 1967). Particles must be above 10 microns to be successfully removed from the lung by ciliary action (Newhouse, 1976).

The possibility of smaller NPV's invading the organism (e.g. via macrophage ingestion in the alveoli) must not be totally ignored. Of further concern in consideration of alternate host invasion is the possible existence of free virions, the presence of which would obviate the need to dissolve polyhedral protein prior to host invasion by virus.

Another concern about the use of whole polyhedra in aerosolized form may be possible allergic response. There have been many foreign proteins incriminated in causing allergic reactions in the lungs, usually hypersensitivity type III reactions. This type of hypersensitivity involves antigen precipitating antibody complexes. It tends to occur in 50% of the population when exposed to heavy doses of a suitable antigen (Nicholson, 1972). The reaction may vary in severity among different people, but can in some cases lead to chronic fibrotic changes, and accompanying decreased ventilatory capacity (Harrison's, 1974). Hypersensitivity type I reactions, bronchial asthma, may be caused by many of the same antigens, but tend to occur in only 10% of the population. The following is a list of sources of foreign materials which have been implicated in hypersensitivity reactions of the lung, to give an idea of their variety: various moldy vegetable materials (hay, sugar cane, maple bark dust, red wood dust, paper), mushroom dust, oak bark, cork dust, various bird droppings, wheat flour weevils, and detergent powder (enzyme detergents only) (Nicholson, 1972). Inhalation tests of the

NPV's of Heliothis zea (Boddie) and Heliothis virescens (Frabricius),
have been done on guinea pigs, with no ill effects observed (Ignoffo,
et al., 1965). Guinea pigs are subject to some of the same ailments
as humans, including hypersensitivity to various foreign substances;
but how good a predictor they are of human reactions to specific
proteins is not known. Certainly there may be allergenic properties
to an NPV insecticidal spray if the requirement of purity of product
is not fulfilled, and the spray includes small amounts of bacterial
material or insect remains. It is accepted that many insects,
including the gypsy moth, have allergenic properties in man (Etkind,
1976). Perhaps, though, problems from aerosol spray would occur only
for the personnel involved in the spraying. They could certainly be
protected with masks, gloves, and other suitable apparel. Before
wide spread use of aerosolized foreign protein is attempted anywhere,
its possible effects should be thoroughly investigated by experts
in several fields (e.g., ecology, medicine, wildlife pathology).
The use of any viral insecticide should be well planned to allow
as brief an application as possible, at a time when viral action can
be fastest and most effective.

A twofold interest has engendered the design of the current
study. First, the choice of subject matter has been governed by the
fact that the viral disease under examination is actively being
considered for biological control of a pest. If a virus is to be

used by man as a tool in the environment, man should have a
thorough knowledge of its character.

Second, even an insect may give clues about the operation of
disease in man. The example of the first experimentally proved pathogen
is one of broader application of knowledge derived from insect
pathology. Exact mechanisms of operation of pathogens in insects
cannot be applied to humans, because in few instances is it possible
to draw analogies between warm and cold blooded animals. But in
general areas, because of some quirk of detail, or because of some
simpler attribute, like a one-food diet, some things may be at first
easier to see in a creature like an insect. An example of current
work on a cold-blooded animal that is being applied to general concepts
in physiology and pathology is work on the role of fever in disease,
using lizards. Apparently, when sick, they actively seek a warmer
environment, and by raising their own body temperatures, create
conditions less optimal for the infecting organism (Bernheim, et al.,
1976).

It may be that more knowledge about human stressors can be
gained from studying insect stressors. In man, innumerable factors
must be weighed in endeavoring to demonstrate environmental influences
in disease. Clues about how to approach an environmental study may
be derived from an attempt at such a study in a laboratory animal.
The definitions of stressors are the same for insects and humans.
It is doubtful that details of operation of stressors can be compared,

but the overall scheme for operation of stressors (albeit different
stressors) may be the same. For example, in man stressors in some
cases probably operate on the immune system. Insects also possess
an immune system, and although not well understood, it appears to
be a sophisticated one. Certain humoral factors, antisomes, in
hemolymph involved in pathogen recognition are not similar chemically
to mammalian antibodies, but may have an analogous role (Anderson,
1975). Anderson has also proposed that insects may possess a
complement system at least partially related to the one in man (Ibid.).
Damage to these chemical systems, possible defense mechanisms, could
increase insect disease. If understood, such damage could lead to
better understanding of how man's humoral defense mechanism might be
damaged by certain stressors. The phagocytic components of insect
hemolymph, the hemocytes, are better understood. This may be because
study can in part be done by direct observation of them. The hemocytes
have been compared to mammalian monocytes, which, of the various known
vertebrate phagocytes, they most closely resemble, morphologically
and physiologically (Ibid.). One stress factor known to affect hemocyte
action is temperature. Jones (1975) describes how Metalnikov in the
1920's observed that at $10^{\circ}C$, hemocytes failed to phagocytize bacteria;
while at a higher temperature they became quite efficient at that job.
Indeed, in many instances, hemocytes seem more voracious than monocytes
(Ibid.). They are usually observed to destroy the bacteria they
engulf, although not always. Failure of cockroach (Blaborus craniifer)
phagocytes to kill some intracellular bacteria has led to the
proposition that those pests may be potential disease carriers (Anderson,

1975). Damage to phagocytic cells may be a common modus operandi

for certain stressors. Although the stressors in vertebrates and

invertebrates may often be different, general patterns may be noted.

Certainly, if the phagocytic cells have similar physiology and bio-

chemistry, similar internal stressors may influence all of them.

The Gypsy Moth Problem

The gypsy moth was introduced into North America from France

in 1868 or 1869. The exact details of the introduction are disputed

but the moths' escape was apparently due to carelessness on the part

of a researcher. They were soon widely dispersed in New England;

and have recently continued to increase their range by attaching

themselves to motor vehicles and being carried to new wooded areas.

Larvae can be a great nuisance in an area suffering from a

high density infestation. They crawl through homes, spoil recreational

sites, and contaminate water with their frass (Leonard, 1974). The

gypsy moth larva has been considered by some the "most injurious

insect pest of hardwood forest trees in New England", and in many

years has defoliated thousands of acres of forest stands: action which,

although not necessarily causing death of infested trees, does inhibit

growth for at least a few subsequent years (Friend, 1945). On the

other hand, some foresters have implied that the larvae may be a

cosmetic problem only. Most recently, a researcher in upstate New

York, Mr. Daniel Smiley, has proposed that after its century of

inhabiting the Northeastern U.S., the gypsy moth has been incorporated
into the food chain. He feels that a sharp cut in the moth's population
would drive away at least one currently important predator, the
calasoma beetle, itself imported as a control agent. The scarce gypsy
moth population would be left to rebuild itself without any inter-
ference from that important regulatory predator (Smiley, 1975).

At any rate, gypsy moth control is a well established activity
of the forestry and agriculture services of state and federal govern-
ments; and a popular activity at that, as anyone who has experienced
an infestation of P. dispar retains little fondness for the cater-
pillar. Controls by man have consisted mainly of the use of pesticides,
but most work has been aimed at biological control agents, including
bacteria and viruses.

Naturally occurring P. dispar predators include several forest
rodents in addition to the imported calasoma beetle mentioned above
(which has successfully established itself as a predator, but has
failed to prevent severe infestations). There are also egg and pupal
parasites, at least some of which have been imported. Six exotic
parasitoids were released in New Jersey in 1971 and 1972 (Leonard,
1974).

What appears from field observations to have the greatest
effect on morbidity and mortality in P. dispar populations are
bacterial and viral pathogens (Leonard, 1974). An epizootic can
end a gypsy moth infestation very dramatically, and has often been

observed to do so (Aruga, 1963; Campbell, 1963; Tanada, 1976). The victims of epizootic mortalities are observed to harbor mainly two pathogens: the bacterium Streptococcus faecalis and the nuclear polyhedrosis virus of P. dispar. Infections with the latter, which seems the most important factor in causing the epizootics, is the point of focus of the present study, and will be dealt with at length. The other major pathogen will be discussed first.

S. faecalis is part of the normal flora in mammals, including man, but may under some circumstances induce disease. At least one strain isolated from the gypsy moth, a motile one, has been shown to be serologically different from those strains found in mammals. (Doane, 1970a). There is a disease of P. dispar named by some "dessication" (Campbell, 1963) in which the larvae develop a diarrheal discharge, lose great amounts of body fluid, and when they succumb are left dry and stiff. Later observors linked these signs with the presence of S. faecalis (Doane, 1970b), and it has since been surmised, after duplication of the disease in laboratory larvae, that the cause of the original "dessication" was in fact S. faecalis infection.

It has been postulated that in the induction of an epizootic there may be some synergism between S. faecalis and the other major gypsy moth pathogen, the NPV (Doane, 1970a). This virus (Fig. I), in the genus Baculovirus, is of a type which has been described in larvae of certain Lepidoptera and Hymenoptera (specifically in the family Tenthredinidae, the sawflies), as well as in one species

of Neuroptera (Bergold, 1963a; Mazzone, et al., 1976). The virus
forms inclusion bodies in steps not yet completely understood.
Virions multiply within the nucleus of the infected cell, and a
protein matrix is formed which encloses a variable number of particles.
When enough such particles have formed, the nucleus and cell are
broken down, and the "polyhedral bodies" are released into the
hemolymph. In P. dispar, polyhedral particles may vary slightly
in size. The size of the polyhedra in other species has been
estimated (with the aid of electron microscopy) as 0.5 - 15 um
depending on species; although exact size may be altered somewhat
during preparation for microscopy (Bergold, 1963a). The size and
nature of the polyhedral bodies make them visible by phase contrast
microscopy, and it is this characteristic which makes it possible to
diagnose insect disease from the virus, and to detect the virus in
insect preparations and tissue culture.

The protein matrix of P. dispar NPV has not been completely
analyzed, although three proteins may be contained in it, as
determined by electrophoresis (Padhi, et al., 1974): one of molecular
weight 29,000; one of MW 18,000; and one of 11,000 MW. The latter
two may be subparticles of the larger protein, although this has not
at present been determined. The protein seems to be held together
with a silica matrix. The matrix is stable only between pH 5 and
pH 8.5 (Bergold, 1963a). Its dissolution at more extreme acidity or
alkalinity explains its means of host invasion. The first steps
in cell infection by an NPV, as studied in the tortoiseshell butterfly

(Aglais urticae L.) include dissolution of the polyhedral protein in alkaline gut secretions, possibly assisted by proteolytic enzymes, and setting virions free to invade gut columnar cells (Harrap, 1970).

Polyhedra (the polyhedral bodies) of at least one Lepidoptera, the western hemlock looper Lamdina fiscellaria lugubrosa are somewhat inactivated by ultraviolet light (Morris, 1971); but in P. dispar, at least, they are known to persist in the environment (on tree trunks, in matted forest floors, protected by bark and decaying vegetation) (Doane, 1975). The polyhedra are first introduced into the forest environment by dying and dead infected larvae. The effect the virus has on the insect is optimal for virus release into the surrounding environment. The dead insects appear almost liquified. They are left in a semi-fluid state, cells broken down and body fluids spreading around them. The carcasses may be found hanging from leaves and branches, forelegs pasted down (Glaser, et al., 1913). This characteristic appearance invoked the term "wilt disease" of the gypsy moth.

Incidence of polyhedrosis (the disease caused by NPV, as it is now called) has been linked by many researchers with density of the larvae. In areas of low density of larval populations very few cases of polyhedrosis are noted; while epizootics (where there is a very high incidence of disease) occur when a population has become very dense (Aruga, 1963; Campbell, 1963; Tanada, 1976). Both the virus and the gypsy moth populations have a tremendous propensity for increase in numbers. As has been mentioned, an epizootic is often

the factor which cuts short an infestation of the moth.

There are several theories, some with better supporting evidence than others, about how the virus is passed from one generation to the next. Virus can persist in forest floor debris for at least several years following an epizootic. Thus some infection may occur when a caterpillar eats vegetation which may have some of this remaining virus stuck to it, (perhaps after being splashed onto the vegetation by rain). This physical method for virus spread has been experimentally demonstrated in at least one species of insect; and has been described in others (Bird, 1961; Jaques, 1964; Magnoler, 1974).

Some researchers feel that there may be some transovarial transmission of the virus, from adult to embryo, virus then being present inside the egg. While there is evidence that other viruses in other insect species may be transmitted this way, evidence is limited in the case of NPV's and CPV's (Tanada, 1976). It has been postulated that this virus may be in an inactive form; or the disease may be in a latent state in the subsequently hatching larvae. (The terms "inactive" and "latent" will be defined presently). No proof has as yet been put forth for this means of transmission in the gypsy moth.

Playing the largest role in vertical transmission of P. dispar NPV is transovum transmission (Doane, 1969). It is not certain whether the polyhedra may actually become embedded in the egg surface during egg formation, but it is generally accepted that hairs and

other debris holding an egg mass together may provide a potent
source of infection. In the laboratory, a percentage of larvae
hatched from eggs collected in the field die from polyhedrosis
during the first instar stage of development. The actual percentage
depends somewhat on the status of the disease the previous year in
the area where the eggs were laid. Usually, when the larvae are
reared individually in the laboratory, there are no further deaths
from polyhedrosis after the first instar. It is postulated that in
the field, the rate of first instar mortality partly determines the
later mortality. As stricken first instars die they may be attached
to leaves and thus polyhedra released by them may subsequently be
consumed by older instars. Convincing evidence for the theory of
transovum transmission has come from work by Doane (Ibid.), who has
demonstrated a significant reduction in first instar mortality
when, prior to hatching, eggs are cleaned of hairs and other debris
and disinfected in solutions of sodium hypochlorite and detergent
(destructive of NPV).

A key question at present is whether or not that is the only
means of vertical transmission (first instars succumbing secondary
to ingestion of polyhedra mixed with egg masses and forest
debris; and later instars becoming infected by eating foliage contam-
inated by first instars). If, in addition, there is transovarial
transmission, then virus may indeed be present in inactive form,
perhaps somehow incorporated into chromatin material, and thus not readily
visible, as it is in the polyhedral stage. Such virus could under

suitable conditions, as in the presence of a stressor, produce disease.

At this point, a brief summary will be given of various terms used to describe viruses, as they are intended to be understood here, and as they have been used by various insect pathologists and virologists. Occluded and nonoccluded refer to the state of the virions in reference to the polyhedral protein matrix, occluded describing the virions enclosed in the polyhedral body, nonoccluded referring to the free virions (Mazzone et al., 1976). Occult, not to be confused with occluded, refers to an inactive virus. Inactive describes the state of a virus present during a latent infection. Latent will be used here as defined by Aruga, (1963): to describe "cases of viral infection where the infectious particles cannot be detected and in which the actual state of the virus cannot as yet be ascertained".

Whether or not NPV's may be involved in latent infections in insects has not been determined, although several authors have suspected or considered their existence (Steinhaus, 1958; Steinhaus, 1960a; Doane, 1976a). Longworth and Cunningham (1968) have experimented with certain foreign substances which when fed to insects have brought on, or apparently "activated" a viral disease native to that insect, but of which the insect was free prior to ingestion of the foreign substance. The substances administered in one experiment consisted of polyhedral protein from another insect. The fed polyhedra were serologically distinct from polyhedra known to cause infection in the test insect; and were also serologically distinct from the virus subsequently causing infection (which turned

out to be the same as the test insect's native polyhedra). The
normal hosts of both these viruses were lepidoptera, one of
which was P. dispar. Similar findings -- appearance of native
infection from an unknown source -- have occurred on feeding Tobacco
Mosaic Virus to hymenoptera. It was assumed in both cases that the
native infection had been in a "latent" form.

If some insect viruses can exist in this state, and trans-
formation can occur at times from latency to frank disease, (i.e.,
activation of an occult virus), then transformation may perhaps occur
not only in the presence of artificially introduced activators as
described above, but also in the presence of internal and external
environmental stressors. The latter may alter tissues physiologically
in some way (Aruga, 1963), thus making the insect tissues and cells
optimal for division and growth of an occult virus. The interest in
this study is in stressors, and it must be added here before pursuing
that subject further that in apparent induction of viral disease
by stressors, no distinction can be made, without proof of foreign
DNA being present in cells, between disease stemming from activation
of an occult virus; and that stemming from sudden proliferation of
a virus which was simply present in insect tissue in very low
concentration. (Actually, the latter case may fall within Aruga's
criteria for the virus in a latent infection; where infectious
particles cannot be detected and the state of the virus cannot be
ascertained).

Several stressors have been suggested in the initiation of insect viral disease. In some instances the stressor may have an effect on the pathogenic organism itself. This type of environmental effect has been named by Steinhaus (1958) an "incitant" effect, rather than a stressor effect which weakens the host organism. The incitant effect would tend to enhance the power of the pathogenic organism.

Temperature is one such factor which may have an apparent effect on viral action, independent of any stress engendered in the host. In cell cultures of Spodoptera frugiperda, there was found to be an optimal temperature for viral activity at 27°C (Knudson, et al., 1974). In an in vitro study of Lymantria dispar L. in larvae fed constant doses of NPV, mortality from polyhedrosis increased with temperature up to 26.5-27.5°C, after which no increased mortality occurred (Yadava, 1970). In these two cases it is likely that there is a biochemical explanation for the relationship of viral activity with temperature. For example, the enzymes involved in viral replication may have a maximum operating capacity at the optimal temperature determined in the lab studies.

Certain chemicals administered to laboratory populations have been felt to trigger latent NPV infection. Yadava (1971) again working with L. dispar L., found that at least under some conditions boric acid (1%), Na_2SiO_3, and certain plant ashes, could shorten the time between administration of infective NPV and appearance of disease. Even though the presence of latency is not proved in his experiment, certainly this may be categorized as an example of stressor

action speeding up an infection.

Another factor implicated by some authors as a possible stressor is nutritional status (Steinhaus, et al., 1960; Pimentel, et al., 1962). Poor nutrition could upset biochemical and physiological balances within an insect, and lead to increased disease. However, whether or not this can have any influence in viral disease is yet to be determined. So far, evidence is in the negative direction. One study which tested effects of inadequate feedings as well as total starvation, demonstrates increased mortality in poorly fed and starved groups, but there was no increased incidence of polyhedrosis. The increased mortality was either of undetermined cause, or due to other pathogens (e.g., S. faecalis) (Pimentel, et al., 1962).

The potential stressor to be examined in this study is high relative humidity. Several observors of field populations of gypsy moths have noted a relationship of increased incidence of polyhedrosis with elevated humidity. Wallis (1957) observed the onset of an epizootic a few days following the beginning of an unusually humid period in the summer of 1956. Glaser, (1915) suspected a similar relationship. Pimentel and Shapiro, (1962) reviewing this subject note that Acqua in 1930 had observed a similar effect of humidity in silkworm infection. Campbell and Podgwaite (1971) in studying gypsy moth populations in different terrains, determined that larvae in a low, marsh area seemed to succumb to polyhedrosis at a greater rate than their counterparts inhabiting higher, drier vegetation. Observors of another species, the California oak moth (Phryganidia californica Packard) led to the conclusion that viral disease of that

moth was favored by the combination of high temperature, high relative humidity, and crowding of the population (Harville, 1955). Steinhaus (1960b) also seems to assume that moisture has a stressor effect on certain insect viral diseases.

However, the field observations on the gypsy moth and on other species have not been duplicated in laboratory experiments. Yadava (1970) raised larvae at three different temperature ranges and three different relative humidities (using all possible combinations of each temperature with each humidity). The larvae were raised in batches of six or eight in Petri dishes, and humidity controlled by use of various salts placed in the dishes. The animals were fed equal amounts of purified NPV polyhedral bodies. While Yadava did find an effect of temperature on mortality, he noted no difference in morbidity or mortality in individuals raised at different humidities. Pimentel, et al., (1962) also found negative results in a similar study of the greater wax moth (Galleria mellonella, L.). Wallis (1959) designed a study to determine whether the gypsy moth might have some preference for dry versus wet sites. This too gave negative results: larval migration was influenced by food and light but not by moisture.

One instance of an insect virus being affected by humidity has been recorded in a laboratory study. Diagnosis of a viral infection of the citrus red mite, Panonychus citri, is based on the presence of birefringent crystalline inclusions within the bodies of the mites. Formation of these inclusions is inhibited by high humidity (Reed, et al., 1974). However, the incidence of infection itself was not

noted to be affected by humidity.

While the current general consensus is that relative humidity probably has little effect on viral activity (Tanada, 1976), the discrepancy between field and laboratory experiments has not been adequately explained. The question remains whether high humidity may in fact be a factor influencing occurrence of polyhedrosis in field populations of P. dispar. It has been postulated that increased moisture, in an animal perhaps lacking efficient mechanisms for water elimination, could provide physiological stress activating a subacute infection (Wallis, 1957). Although Yadava's work indicated no effect of humidity on the invasive and infective properties of ingested polyhedra, that does not disprove an unexplained effect which may in some conditions stimulate activition of an occult virus. Such an effect might have been masked in the experiment where virus was fed to the larvae, insuring a substantial base level of infection. Also we have no information (and at the present time such information would be difficult or impossible to obtain) about the possibility that latent infection may exist in some populations and not others.

Design of Current Study

A humidity comparison experiment was attempted. P. dispar eggs were used which came from an area that had a known "moderate" level of polyhedrosis during the summer prior to the collection of the eggs. Larvae were hatched from sanitized eggs to allow minimal or no exposure

to the NPV of P. dispar. The effects of high relative humidity on
appearance and expression of nuclear polyhedrosis was determined.
As an adjunct, the effects of a liquid rich diet on the appearance
of polyhedrosis in this population was also determined.
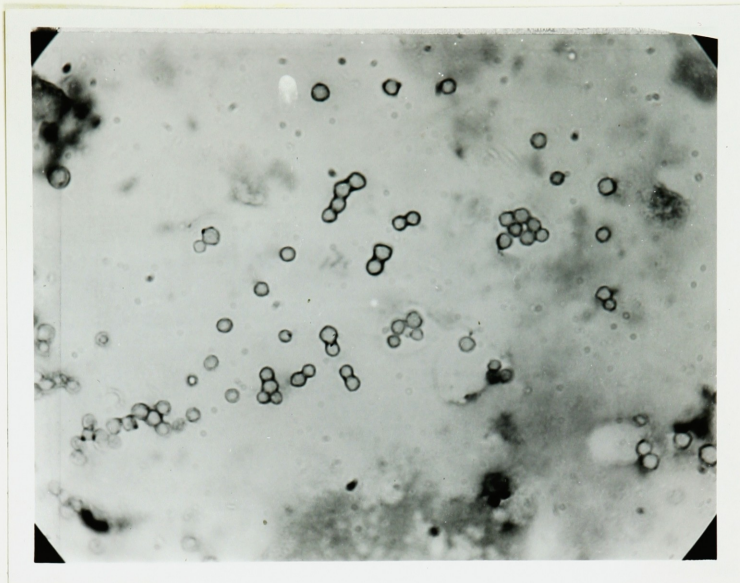
FIG. Ia. Polyhedra of the nuclear polyhedrosis virus of
Porthetria dispar, as seen with phase-contrast microscopy.
(Magnification 1700 x).*
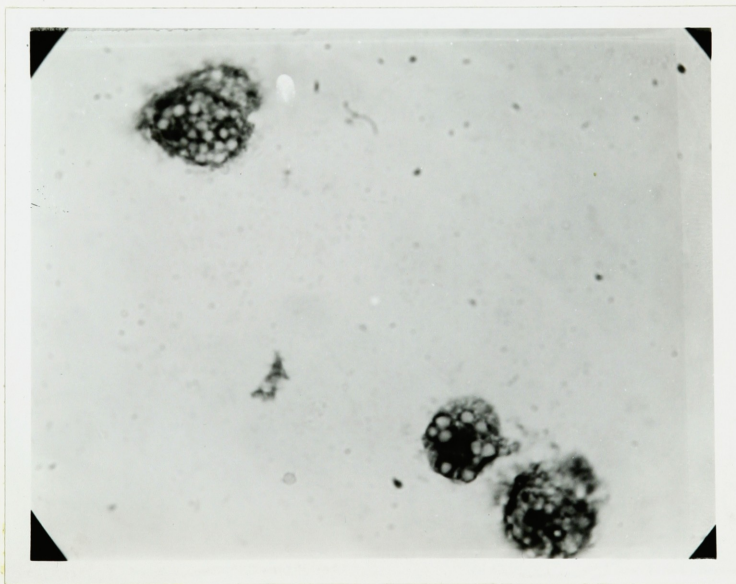
FIG. Ib. Blood cells of Porthetria dispar, containing numerous
polyhedral bodies. (Magnification 1700 x).*

*Courtesy of the U.S.D.A. Forest Service Laboratory, Hamden, Ct.

MATERIALS AND METHODS

Egg masses of <u>Porthetria dispar</u> L. were collected from the
Brooklyn area of eastern Connecticut. Fifteen of these egg masses
were combined for use in this experiment. The masses were broken
up and the eggs vacuum-cleaned to allow removal of hairs, dust, and
other debris, with minimal damage to the eggs. Viable non-
parasitized eggs were then selected with forceps, and fastened
inside one-inch strips of gauze tubing, in groups of approximately
200. These packets of eggs were "sanitized" with solutions of
<u>Clorox</u> (sodium hypochlorite) and a detergent, <u>Triton-X</u>, according
to the method described by Doane (1969).

After cleaning, when dry, the gauze packets were cut open and
eggs counted. Approximately 50 eggs were placed in each of 28
sterile Petri dishes, using sterile technique. Five hundred sixty
such individual eggs were divided into four groups of 140; and those
were subdivided into seven groups of 20. Each group of 20 dishes
was held in a clear metal basin.

The four main groups were treated as follows: two were kept at 80° F
in the high-humidity hatching room, (see Fig. II for humidity ranges),
where one was fed California <u>Iceberg</u> lettuce and the other fed a
previously tried artificial diet (see Tables I and II for ingredients).
The other two groups of 140 were moved to an adjoining lower humidity
room. Again, one group was fed lettuce and the other artificial diet.

This room had the same ambient temperature as the high-humidity room; but instead of having a humidifier it was equipped with a small fan. This generated a slight air current which insured a relative humidity (R.H.) between 20 and 30 % (see Fig. II). As soon as larvae were too large to crawl through, holes were punched in the tops of the Petri dishes in the low R.H. room, to insure a maximally low humidity in these dishes. Fluorescent lighting was provided during the same intervals in both rooms.

A few extra caterpillars were allowed to hatch and were placed in individual Petri dishes, some with lettuce and some with artificial diet. Then the dishes of eggs were refrigerated to prevent further hatching.

After about one week many first instar larvae raised in the low humidity rooms on artificial diets died. Few deaths were noted in the high R.H. room or in the low humidity - lettuce fed group. The dead first instar larvae appeared dessicated, and it was surmised that drying of food in the low humidity room (which occurred much faster than in the high humidity room), together with the small size of the first instar larvae, was the cause of this selective high mortality. Therefore, all first instar larvae were returned to the high humidity room for two days, to allow remaining larvae to recover and grow a little larger.

The dead larvae were kept in their individual Petri dishes and frozen for later examination and determination of cause of death. Depleted groups were replenished with the extra caterpillars that had

been hatched earlier. Also, the dishes of extra eggs were removed from refrigeration, and enough caterpillars were hatched to bring the count in each group to over 120.

Each individual caterpillar was examined and fed every other day, using methods as sterile as possible, to prevent any transfer of viral infection between individuals. Artificial diet was administered in half-inch cubes. Lettuce was cut into 1" x ¼" strips, and rinsed briefly in sterile distilled water.

The artificial diet used was of two types. Four of the groups on artificial diet received a formula obtained from Bio-Serv, containing the antibiotic Aureomycin (chlortetracycline hydrochloride) (see Table I for ingredients). The rest received diet prepared without antibiotic at the Connecticut Agricultural Experiment Station (C.A.E.S.) (see Table II) (Leonard, et al., 1966). Lettuce was obtained at a local grocery store. Although it was not guaranteed to be free of polyhedral bodies, it was assumed doubtful that the lettuce would contain polyhedra specific P. dispar. Lettuce is not a natural food of the gypsy moth, and it would be unlikely to find natural populations of the moth in areas where lettuce is grown.

Individuals were examined for stage of development and general appearance. Petri dishes were cleaned as necessary and changed if fungus appeared. Caterpillars without movement were listed as dead, and their general appearance was noted. They were kept in their Petri dishes, and frozen for later examination. When pupae appeared

they were also frozen. After six weeks most larvae had developed
to the fifth and sixth instar, and the study was terminated. Twenty
individuals from each of the four groups were selected to metamorphose,
mate, and produce eggs. Each group of 20 was placed in a battery
jar with a gauze cover, two jars in each rearing room (one in each
room with the artificial diet, and one with lettuce). After one week
all jars were placed together in the humid room. These larvae were
to be used as a source of eggs for further study, and were observed
for a general impression of the ability of individuals from each of
the four groups to continue normal development.

The remaining healthy larvae and pupae were frozen, and along
with the previously frozen cadavers were examined in two ways.
First, a squash slide preparation was made of hemolymph and some
insect tissue for examination under phase contrast microscopy to
determine the presence of polyhedra. Then, as an additional check,
macerated larvae were suspended in a small amount of distilled water
in test tubes, allowed to decay and settle for a few weeks. The
sediment at the bottom of each tube was examined with phase contrast
microscopy for the presence of polyhedra.

Controls:

Twenty-five larvae were hatched from unwashed eggs and reared
at 80°F. at uncontrolled ambient room R.H., on a different floor
from the experimental group. These were all fed artificial diet and

were cared for and examined as described above.

For the basic level of disease in the population, data from egg masses raised at the C.A.E.S. were used. These were egg masses from the same collection as the ones utilized in this experiment. These egg masses, unwashed, were grouped in batches of two. About 20 larvae were reared from each of these mixtures, in individual cups on artificial diet without antibiotic (see Table II) at 72°F. and ambient humidity. They were maintained through the third instar. Larvae dying in the first and second instars were examined under phase contrast microscopy for the presence of polyhedral bodies.

Figure III presents a diagramatic outline of the experimental design.

Percent
Relative
Humidity

Days after first hatchings.
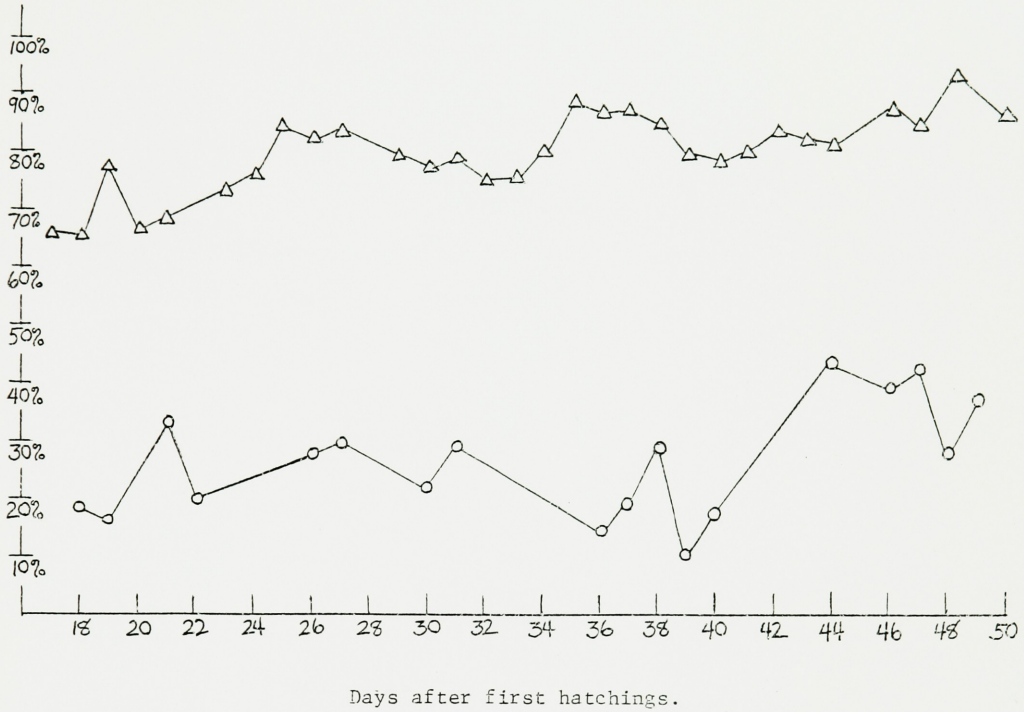
o———o Low Humidity Room

△———△ High Humidity Room

FIGURE II. Sample recordings indicating differences in Relative
Humidity Ranges in high and low humidity rooms during
4 1/2 weeks of experimental rearing of Porthetria
dispar larvae.

FIGURE III. Flow Chart of Experimental Design.   40

FIGURE III. Flow Chart of Experimental Design.
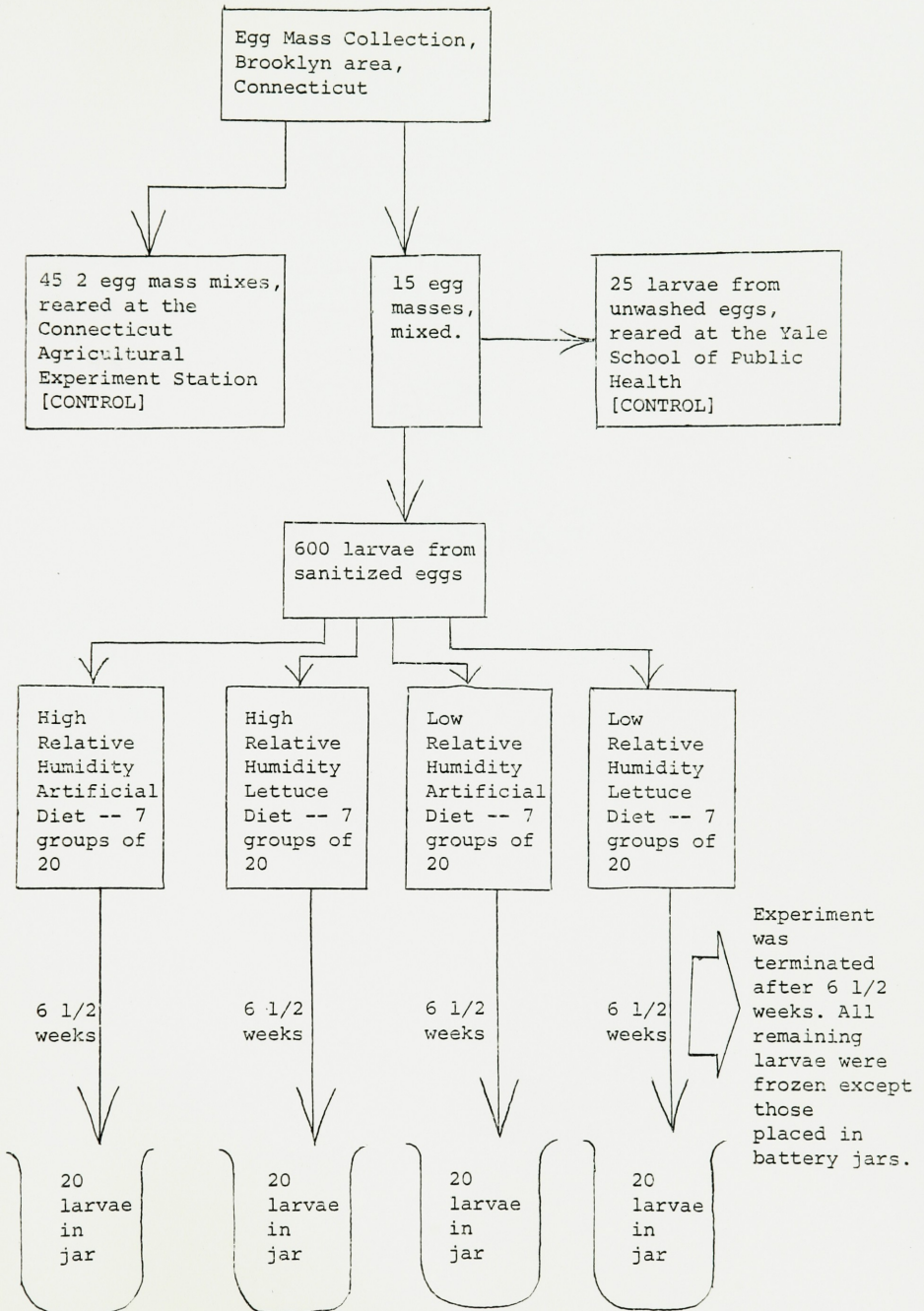
Table I.   Gypsy moth rearing media with antibiotic

            Contents: Agar
                          Ground wheat germ
                          Cholesterol
                          Salts
                          Sodium propionate
                          Locust Bean Gum
                          Cellulose Fiber
                          Sorbic Acid
                          Ascorbic Acid
                          Aureomycin
                          i-Inositol
                          Casein
                          Methyl para-hydroxybenzoate
                          Fructose
                          Choline Chloride
                          Linolenic Acid
                          Methyl Linoleate
                          Vitamin mix (Vanderzant Modified).

                          Taken from manual from
                          Bio-Serv, Inc., Frenchtown,
                          New Jersey

Table II.   Gypsy moth rearing media without antibiotic

            Contents: Water (distilled)
                          Casein, vitamin free
                          4 M potassium hydroxide
                          Salt mixture, Wesson W
                          Sucrose
                          Fructose
                          Wheat germ meal - Type A
                          Choline Chloride
                          Methyl para-hydroxybenzoate
                          Agar
                          Linolenic Acid
                          Ascorbic Acid
                          Vitamin solution (Niacin, Calcium
                             pantothenate, Riboflavin, Thiamine
                             hydrochloride, Pyridoxal hydrochloride,
                             Folic acid, Biotin, Vitamin B-12).

                          Described in Leonard and
                          Doane, 1966; supplied by
                          C.A.E.S., New Haven.

RESULTS

First Instar Mortality

Mortality occurring during the first larval instar in the
original 28 groups of 20 hatched from sanitized eggs is shown
in Table III. The 82 dead first instar larvae appeared dessicated.
The majority of deaths occurred in the low-humidity artificial
diet group. Each larva was examined microscopically, using a
squash-slide prepared with distilled water as a suspension medium.
No polyhedral bodies were noted on any of the slides, either
free-floating or contained within cells of tracheal or other tissue.
Occasional bacteria (diplococci and bacilli) were noted on about
half the slides examined. The diplococci, although not identified,
were suspected to be Streptococcus faecalis, the bacterial pathogen
most frequently found in larvae of the gypsy moth (Doane, 1970b).

First instar mortality in controls. The fate of first instar larvae
in the control groups was somewhat different. In the single control
group of 23, raised in a laboratory room without heat or humidity
controls, on artificial diet, only three (13%) first instar deaths
occurred. The microscopic findings in two were the same as those
for most of the experimental group: no polyhedra were present and
bacterial counts were low or absent. The other larva contained
numerous polyhedral bodies. With phase contrast microscopy these
appeared to have the characteristic shapes associated with the NPV

that is customarily noted to infect P. dispar (see Fig. I). However, verification of their identity with electron microscopy or with serologic studies was not within the scope of this investigation.

Data from the larvae reared from mixed batches of unwashed eggs at the C.A.E.S. is contained in Table IV. These groups of larvae were maintained through the third or fourth instar, at a room temperature of 70-72°F., at ambient humidity. They were fed the formula of artificial diet outlined in Table II. First instar victims of polyhedrosis were diagnosed by examination of a squash-smear, with phase contrast microscopy. No mortality was reported following the first instar in these groups.

The 4.3% polyhedrosis mortality in our single control group is within the range obtained in the groups at the C.A.E.S. All the eggs reared there and in this laboratory came from the same source. The rate of NPV-caused mortality in the control group of this study was consistant with that from the C.A.E.S., and indicates that mixing several egg masses together did not significantly affect the rate of NPV disease.

Data from the control groups indicates that there was a significant level of NPV in this population. This was corroborated by field observations in the Brooklyn area of Connecticut for the summers of 1975 and 1976, preceding and following our egg collection. In both years there was a moderate occurrence of wilt disease in the P. dispar population from which the eggs for this study were gathered (Doane, 1976b).

It is significant that in the 28 experimental groups of 20, no polyhedral deaths occurred in the first instar. This is an indication of the effectiveness of the egg sanitization method (Doane, 1969) here employed.

Development of Larvae Beginning with Instar 2 (L-2)

The rest of this presentation deals with the results of the 28 groups of larvae maintained from the 2nd instar on. Because of high 1st instar mortality in some groups, replacements were hatched from left-over sanitized eggs. Inadequate numbers of replacements were available, and so some groups contained less than 20 individuals.

Development and growth. Individuals in different groups developed at different rates, attained different sizes, and reached pupation at different times. The average times from hatching to first molt are presented in Table V. Weight ranges and average weights of sample individuals from the four major groups are given in Table VI. The percentage of larvae in each group reaching pupation within six weeks of the date of hatching are listed in Table VII.

These data show that the lettuce eaters in the humid room developed at the slowest pace. Both groups of lettuce eaters attained about the same weights. The average weight of larvae in the low-R.H. artificial diet group was double that of those in groups fed lettuce; and the average weight of larvae in the high humidity artificial diet group was about double that of their low-R.H. counterparts.

Three logistical problems were encountered in this experiment. The first was drying of food, in the low-R.H. room. It was not possible in these groups to administer large amounts of food, as that tended to raise the humidity in the Petri dishes. The small amounts given were either devoured or dried out in the 48 hour period between feedings. A second logistical problem was the appearance of fungus in dishes in the humid room. In the artificial diet group this was a black fungus, unidentified (see Table IX). A third logistical problem was the occurrence of "wet rot" in the lettuce of 76% of the high-R.H. lettuce diet group, resulting in semi-liquification of the leaves.

Morbidity and Mortality after the 1st Instar (L-2 to Pupation).

Morbidity. The only definite sign of illness in living larvae, noted by us, was a diarrheal discharge noted in some. This was more prevalent among those in the humid room. Smears were not done to look for pathogens in this frass (because this study was concerned with the disease caused by NPV, which does not characteristically produce diarrheal frass, and in which polyhedra are not character-istically secreted from the gut).

Mortality. Table VIII indicates overall mortality (irrespective of cause) occurring in each group, by instar. It does not include data on four mortalities, which occurred after the larvae had been transferred to battery jars, at the termination of the individual-

rearing phase of the experiment. These four will be discussed
subsequently.

Of the deaths from individual Petri dishes, none at death had
the usual signs of nuclear polyhedrosis (disintegrated, "melted"
appearance, falling apart when touched). Some were flaccid and wet,
but when they were moved they were able to retain their structure.
Especially in the high relative humidity lettuce diet group, the
wetness was later considered of external origin, and not a sign,
as was at first suspected, of leaking body fluids. Other larvae
appeared quite leathery or rubbery, and dry.

None of these dead larvae were diagnosed as having died from
nuclear polyhedrosis. (One was lost to examination -- a 2nd instar
from the low-R.H. lettuce diet group. However, on gross examination
this had not been found to have the characteristic signs of wilt
disease). On two initial smears of larvae from the high-R.H.
lettuce diet group (one from group 9 and one from group 14)
refractile particles were noted which were suspected to be polyhedra.
In the smear of the group 14 larva, diplococci were also seen (which
may have been the S. faecalis organisms known to infect P. dispar).
However, after one month of the sedimenting process to which each
larva was subjected, examination of the sediment from each of these
suspect larva (with phase contrast microscopy) showed no polyhedra.
Hence it was concluded that there was no polyhedral death in the
entire group of larvae reared from sanitized eggs.

Some organisms that were present in the smear of dead larvae were diplococci, numerous rod-shaped bacilli (of various sizes and degrees of refractility), and various other unidentified micro-organisms, some possibly yeasts, some protozoans, and some appearing as spore like structures. Whenever a dead larva was discovered it was either immediately examined or frozen, but since each group was checked only every other day, it was possible for mortality victims to go undiscovered for nearly 48 hours, more than enough time for overgrowth by saprophytic organisms.

Ingestion of molds which developed on food substances in the humid room could account for spore-like or yeast-like organisms seen on smears. The relationship between mortality and the occurrence of mold on food is outlined in Table IX. There was no statistically significant relationship between the two (X^2 analysis of independent samples). By similar analysis, no statistically significant relation-ship existed between the occurrence of "wet rot" and mortality.

Examination of Healthy Larvae and Pupae

The occurrence of bacteria noted on smears of healthy larvae ("healthy" meaning alive) is outlined in Table X.

In late instar and prepupal stage larvae, globular like particles were noted. These were thought to be lipid, either breakdown products of the action of **Juvenile Hormone**; or storage materials for the pupal stage of development. Oil red O stain

demonstrated the fat content of these globules. Their significance
to this study is the fact that, since they were refractile and were
present in generally round form in all sizes, it would have been very
difficult to differentiate them from one or two polyhedral bodies.
Thus, from these examinations, no statement can be made about the
absolute presence or absence of a small background level of polyhedra.

Examination of pupae revealed similar findings, with the occasional
presence of diploccoccal like organisms (Table X). Globular particles
were present, similar to those found in late instar larvae. Again,
no statement can be made about background polyhedra. However, the
pupae, (except for two, in groups 9 and 18, which had a yellow color
and failed to harden), had a uniform, healthy appearance.

Findings in late instar larvae from the control groups were
generally the same as those in the test larvae. No potentially
pathogenic organisms were noted in those examined (Table X).

The continued use of an artificial diet containing an antibiotic
in four of the groups (1, 2, 20, and 21) had no effect on mortality
rate in these groups, or on microscopic findings in healthy individuals
(Tables VIII and X).

The following observations were made on the larvae and pupae
set aside in battery jars, at the termination of the experiment,
and allowed to complete their development and produce eggs:

High Relative Humidity, Artificial Diet Group: 19 of the
20 larvae successfully pupated and emerged as adult moths. One

pupa failed to emerge. On microscopic examination no polyhedra or other pathogenic organisms could be detected in this pupa.

Eggs were produced.

High Relative Humidity, Lettuce Diet Group: 19 of the 20 larvae reached adulthood. One pupa failed to emerge. On microscopic examination yeast-like particles (lucent, rounded, not refractile) were observed, but no polyhedra or other pathogenic organisms were present.

Eggs were produced.

Low Relative Humidity, Artificial Diet Group: 19 of the 20 larvae successfully pupated and emerged as adult moths. One late instar larva died -- having a "melted" appearance like that described for wilt victims. On microscopic examination, polyhedra-like particles were seen. The larva was suspended in sterile distilled water, and within one day a gray sediment appeared. Examination of this sediment with phase contrast microscopy revealed polyhedral bodies -- confirming the diagnosis.

Eggs were produced.

Low Relative Humidity, Lettuce Diet Group: 19 of the 20 larvae succeeded in pupating and emerging as adults. One larva died in the prepupal stage. On microscopic examination no polyhedra or other pathogenic organisms could be detected.

Eggs were produced.

See Figure IV for size and general appearance of P. dispar developmental stages (larval and adult).

Table III.

The levels of mortality in the first instar larvae of P. dispar in the initial 28 groups of 20 hatched from sanitized eggs.

Category	Group	Number of 1st instar deaths	% 1st instar deaths
High R.H.,	1.	1	5
Artificial	2.	1	5
Diet	3.	0	0
	4.	3	5
	5.	2	10
	6.	1	5
	7.	4	20
	Total	12	8.5
High R.H.,	8.	5	25
Lettuce	9.	1	5
Diet	10.	0	0
	11.	0	0
	12.	0	0
	13.	0	0
	14.	1	5
	Total	7	5
Low R.H.,	15.	1	5
Artificial	16.	6	30
Diet	17.	3	15
	18.	8	40
	19.	14	70
	20.	11	55
	21.	4	20
	Total	47	34
Low R.H.,	22.	0	0
Lettuce	23.	1	5
Diet	24.	2	10
	25.	5	25
	26.	4	20
	27.	3	15
	28.	1	5
	Total	16	11.4
Control		3	13

Table IV.

Rate of Polyhedrosis caused mortality of larvae from mixed
egg masses, reared at C.A.E.S., 1976*.

Mixed Egg mass group number	Number of Larvae in group	1st instar wilt deaths	% mortality from wilt
1.	5	1	20.0
2.	58	5	8.6
3.	40	9	22.5
4.	40	3	7.5
5.	35	1	2.9
6.	40	6	15.0
7.	40	0	0.0
8.	16	7	43.7
9.	6	1	16.6
10.	15	0	0.0
11.	20	10	50.0
12.	40	8	20.0
13.	42	5	11.9
14.	30	25	83.3
15.	41	13	31.7
16.	19	5	26.3
17.	37	17	46.0
18.	39	6	12.8
19.	40	1	3.2
20.	39	15	38.4
21.	36	4	11.1
22.	40	1	2.5
23.	40	10	25.0
24.	42	25	59.5
25.	22	0	0.0
26.	24	2	8.3
27.	27	0	0.0
28.	29	0	0.0
29.	40	2	5.0
30.	28	8	28.5
31.	27	2	7.4
32.	27	6	22.2
33.	20	0	0.0
34.	32	20	62.5
35.	40	12	30.0
36.	27	3	11.0
37.	30	2	10.0
38.	32	0	0.0
39.	40	5	12.5
40.	18	0	0.0
41.	28	1	3.6
42.	30	0	0.0
43.	44	14	31.8
44.	31	7	19.4
45.	32	7	21.9

n=1418   total deaths=269   total % deaths=19.0%

* Doane, C.C., Unpublished data.

Table V.

Average time from hatching to first molt, by group, measured for the P. dispar larvae, when hatch dates and dates of first molt are known.

Category	Group	n	Average days to first molt
High R.H.,	1.	17	7.5
Artificial	2.	19	10.7
Diet	3.	17	9.5
	4.	15	9.9
	5.	18	10.3
	6.	19	8.5
	7.	16	9.1
	9.4 average days for group		
High R.H.,	8.	14	13.5
Lettuce	9.	18	10.8
Diet	10.	16	12.7
	11.	19	13.3
	12.	20	12.0
	13.	20	11.1
	14.	18	11.0
	12.0 average days for group		
Low R.H.,	15.	18	10.5
Artificial	16.	14	11.4
Diet	17.	17	11.1
	18.	11	9.3
	19.	4	10.2
	20.	7	9.1
	21.	15	11.1
	10.4 average days for group		
Low R.H.,	22.	20	11.8
Lettuce	23.	19	12.8
Diet	24.	18	13.0
	25.	15	11.9
	26.	16	13.6
	27.	14	13.5
	28.	16	14.1
	13.0 average days for group		

Table VI.

Range of weights and average weight of samples of
larvae of P. dispar from each of the four experimental
groups, at age six weeks.

Category	Sample size (n)	Average wt. (g.)	Range of weight (g.)
High R.H., Artificial Diet	26	0.86	<0.002-1.650 g.
High R.H., Lettuce Diet	37	0.26	<0.001-0.589 g.
Low R.H., Artificial Diet	30	0.44	<0.001-1.628 g.
Low R.H., Lettuce Diet	35	0.29	<0.002-0.563 g.

Table VII.

Number and percentage of larval P. dispar reaching pupation
within six weeks of hatching, by group.

Category	Group	Number in group hatched at least 6 wks. before end of expt.	Number pupated	Percent pupated
High	1.	20	12	60
R.H.,	2.	20	8	40
Artificial	3.	20	13	65
Diet	4.	16	9	56
	5.	18	10	56
	6.	18	10	56
	7.	18	7	39
	Total	130	69	53%
High	8.	16	0	0
R.H.,	9.	20	1	5
Lettuce	10.	20	1	5
Diet	11.	20	0	0
	12.	20	0	0
	13.	20	0	0
	14.	19	0	0
	Total	135	2	1%
Low	15.	19	3	16
R.H.,	16.	17	3	18
Artificial	17.	20	4	20
Diet	18.	15	4	27
	19.	16	3	19
	20.	15	4	27
	21.	16	2	12
	Total	118	23	19%
Low	22.	20	6	30
R.H.,	23.	19	1	5
Lettuce	24.	19	2	10
Diet	25.	20	5	25
	26.	19	0	0
	27.	19	0	0
	28.	19	0	0
	Total	135	14	10%

Table VIII.

Larval mortality of P. dispar after the first instar in each group, by instar.

Category	Group	Number in Group raised from 2nd instar stage	2	3	4	5	6	Total Deaths	%
High	1.	20			1			1	
R.H.,	2.	20			1			1	
Artificial	3.	20			1			1	
Diet	4.	16							
	5.	18							
	6.	19					1	1	
	7.	17				1		2	
	Total	130	0	0	3	1	1	5	4%
High	8.	16					1	1	
R.H.,	9.	20			2	6	1	9	
Lettuce	10.	20	1	1		1	1	4	
Diet	11.	20		2	2	5	1	10	
	12.	20		2	2	2	1	7	
	13.	20			2	4	2	8	
	14.	19			4	4		8	
	Total	135	1	5	12	22	7	47	35%
Low	15.	19							
R.H.,	16.	16				1		1	
Artificial	17.	20							
Diet	18.	15							
	19.	15							
	20.	15							
	21.	15							
	Total	115	0	0	0	1	0	1	1%
Low	22.	20							
R.H.,	23.	20							
Lettuce	24.	19	1				2	3	
Diet	25.	20							
	26.	19							
	27.	19							
	28.	19			1			1	
	Total	136	1	0	1	0	2	4	3%
Control		21	1	0	0	0	0	1	5%

Table IX.

Appearance of fungus developing on food, related to mortality of
larval P. dispar in experimental groups.

Group	Total Number	Number with fungus	Number mortalities	Mortalities post fungal exposure
High R.H., Artificial Diet	130	10	6	0
High R.H., Lettuce Diet	135	22	47	7*
Low R.H., Artificial Diet	115	0	1	0
Low R.H., Lettuce Diet	136	0	4	0

$*\underline{x}^2$ analysis of independent samples indicates
no correlation between appearance of
fungus and subsequent mortality.

Table X.

Occurrence of bacteria noted in healthy larvae and pupae of P. dispar in experimental groups.

Category	Group	Number of healthy larvae examined	Number of pupae examined		Total in which bacteria were noted	
High	1.	2	2		0	
R.H.,	2.	4	4		0	
Artificial	3.	2	3		0	
Diet	4.	6	2		0	
	5.	4	1		0	
	6.	4	4	(4**)	4*	(4**)
	7.	5	0		0	
	Total	27	16			
High	8.	12	0		6*	(2**)
R.H.,	9.	5	1	(1*)	4*	(1**)
Lettuce	10.	8	1	(1**)	7*	(7**)
Diet	11.	5	0		3*	(3**)
	12.	5	0		0	
	13.	7	0		0	
	14.	8	0		0	
	Total	50	2			
Low	15.	6	2		0	
R.H.,	16.	7	0		0	
Artificial	17.	5	4	(4**)	6*	(6**)
Diet	18.	5	3		0	
	19.	6	2		0	
	20.	6	2	(2**)	4*	(4**)
	21.	7	1		2*	(2**)
	Total	46	14			
Low	22.	3	5		0	
R.H.,	23.	8	1		0	
Lettuce	24.	6	2		0	
Diet	25.	6	2		0	
	26.	9	0		0	
	27.	8	0		4*	(4**)
	28.	8	0		1*	(1**)
	Total	48	10			
Control		4	0		0	

* All bacteria
** Diplococci

DISCUSSION

Ability to Thrive

From the data on weight, mortality, and rate of development,
it is clear that larvae raised under different conditions experienced
various degrees of difficulty in their abilities to thrive. The
stressor factors (some planned, some unplanned) influencing each
group will be discussed first.

Low humidity, artificial diet. First instars in this group
apparently succumbed to insufficient food, water, or a combination
of the two. Since few pathogenic organisms were observed on smears
no infectious disease was diagnosed as a major cause of death in
this group. Their appearance following death, not the character-
istic "disintegrated" one of wilt disease, is further evidence
that these first instars did not succumb to polyhedrosis.

Older instars in this group were able to survive and develop
but remained very small, one-half the size of their counterparts
in the humid room. This can be attributed to semi-starvation.
Throughout the first few weeks of the experiment it was not
possible to replenish food more often than every forty-eight hours;
and food dried out within twenty-four hours. Efficiency in the
feeding schedule greatly improved halfway through the experiment;
but the effects of early malnutrition left the larvae stunted.

The bacteria present in some of the apparently healthy individuals may be normal flora. It is thought that there may be some strains of S. faecalis (a diplococci resembling those seen on some smears of healthy individuals) which are not virulent for P. dispar (Doane, et al., 1970).

Of those larvae allowed to pupate, reach adulthood, and produce eggs, 95% were able to do so, and many eggs were produced. This is an indication of the basic adequate state of health in this group. However, out of the more than 580 larvae raised in this experiment, the only case of polyhedrosis occurred in this group.

Low humidity, lettuce diet. Fewer animals in this group succumbed during the first instar, probably because lettuce remained moist and edible for longer periods. These larvae remained small, and probably were undernourished as were those discussed above. Lettuce, even in copious amounts, is not an ideal nutriment for P. dispar (Glaser, 1915) and in order to maintain humidity at a low level for these larvae it was not possible to feed them large amounts of the moist leaves. They were able to consume most of their portions in less than forty-eight hours; and any small pieces remaining dried up quickly.

Presence of bacteria in apparently healthy individuals has been discussed.

The later development of this group was similar to that of the artificial diet group in the same room. One larva died of an undiagnosed cause.

In this group, underfeeding did not induce viral disease.

High humidity room, artificial diet. The larvae in this group thrived best, and experienced minimal stress. The humidity kept the food supply moist and edible. The only logistical problem experienced in this group, the occasional appearance of mold, did not affect the survival of the larvae. On the whole this was the most vigorous of the four groups, and had the best pupation rate. Of the 20 individuals followed through later development, only one pupa failed to emerge. No pathogens were detected in this individual. If the humidity level experienced by these animals was a stressor, it was not evident in these conditions.

The presence of bacteria in apparently healthy individuals has been discussed. The small amount of antibiotic (a tetracycline) present in the food of two of the sets of 20 in this group, and in two of the sets of the 20 in the low humidity-artificial diet group, had no effect on rate of appearance of bacteria in healthy individuals.

High humidity, lettuce diet. In weight and rate of development these larvae lagged behind the artificial diet eaters in the humid

room. Besides being a shorter size they appeared thin, relatively hairless, and were sluggish. Whether humidity itself (higher in their dishes than in other dishes in the same room) was a major stressor inhibiting this group's ability to thrive is difficult to say. They had nearly the same degree of malnourishment as the lettuce eaters in the drier room, although lettuce in the humid room stayed edible slightly longer. However, contributing to their malnourishment was the fact that humid room lettuce was very subject to "wet rot" (most likely caused by some species of Erwinia). Once rotten, it was inedible. Also, wet rot caused liquification of the lettuce, and this combined with condensation in the Petri dishes made locomotion more difficult for the caterpillars. Matting of the larvae's hair by the liquid may have interfered with respiration, but this was not closely examined and cannot be stated for certain.

The incidence of mold was greater than in the dishes with artificial diet in the humid room. However, this seemed to have no effect of the morbidity of the larvae. It cannot be said with certainty whether the green mold found here or the black mold found in dishes of artificial diet might be at all pathogenic for the larvae, since the molds were not identified. Occurrence of mold was not linked to subsequent mortality.

The presence of bacteria in living larvae of this group may be more important than in the other three groups, since some later mortality in this group was attributed to bacteria.

Again, in this group, after 20 individuals were placed in the
battery jar, one casualty occurred: one pupa failed to emerge.
The yeast-like particles seen on microscopic examination may be an
indication of a fungal pathogen; but since the pupa was not immed-
iately examined, a fungus may have been a secondary invader.

In spite of the stressors present in the environment in which
this group was reared, no viral disease appeared.

Mortality.

There was no significant difference in mortality rates in
three of the groups (from second instar on): the high humidity
artificial diet group; the low humidity artificial diet group; and
the low humidity lettuce diet group. Mortality that occurred in
these groups was linked with the following microscopic findings:
presence of diplococci in about 60%; presence of rods in a smaller
percentage; no apparent cause for disease in about 40%; and polyhedra
in the single individual in the low humidity artificial diet group.
Of the bacteria seen on smears, the only ones which could be interpre-
ted as probable pathogens were the diplococci, which were suspected
to be S. faecalis, a known P. dispar pathogen. The others were
most likely saprophytic organisms, occurring in cases where dead
larvae were not immediately frozen.

It can be assumed in these three groups that this mortality
was an indication of the survival potential under the laboratory
conditions created in this experiment.

Groups fed artificial diet containing antibiotic. The pre-
sence of Aureomycin (chlortetracycline hydrochloride) in the diet
of four of the sets of 20 had no effect on survival rate. The
tetracyclines act by binding on 30s ribosomes, inhibiting protein
synthesis (Goodman and Gilman, 1975). Whether this action or some
other undescribed intranuclear activity may inhibit NPV replication
is not known. At any rate, such action does not explain absence
of polyhedrosis in these groups, as the 24 groups of larvae with-
out antibiotic also failed (with the exception of one case) to
demonstrate polyhedrosis.

Electron microscopic and serologic studies were not done to
verify the identity of the polyhedra causing the single NPV death.
The shape and size as seen with phase contrast microscopy were the
same as those of the NPV normally observed to infect P. dispar.

There may be several explanations for this single polyhedro-
sis death. Other experiments using the method of egg sanitization
employed here produced a 0.1% rate of polyhedrosis subsequent to
the sanitization (Doane, 1969). Our single case out of about 600
larvae gives an attack rate slightly over 0.1%. However, in the
previous rearings the infections occurred in first instar larvae.
The polyhedral victim in this study was a fifth or sixth instar
larva.

If the source of polyhedra was the egg surface, then disease
in this larva occurred after an incubation period of several weeks.

That length of time, not uncommon in larvae held at lower tempera-
tures (Aizawa, 1963) but unusual at 80°F, might be explained by
very low dosage; by some quality of the larva preventing immediate
viral proliferation; or by some quality of the virus causing it to
exist for a while in an occult state,or a quiet state. All are
matters of speculation. If such a virus was in an occult state,
the later stressor of starvation may have activated it. Other
authors have not noted a causal relationship between undernourishment
and viral disease in insects (Steinhaus et al. 1960). The crowd-
ing encountered by the larva upon entering the battery jar may also
have activated a latent or quiescent infection. Other investi-
gators have noted a relationship between crowding and occurrence
of viral disease.

One week after larvae were placed in the battery jars, all
jars were put into the humid room to prevent further drying of food
and allow maximum egg production. It is likely that viral infec-
tion had begun in this larva prior to introduction of the jar into
the humid room, and that the humidity had no influence on the out-
come of the infection. However, this larva experienced a humidi-
ty factor to which the larvae raised entirely in the humid room
were not subjected: a sudden rise in ambient humidity. Whether
that could have influenced viral infection or not is a matter of
pure conjecture, and could be examined only with further experi-
mentation.

The possibility of an occult or quiet virus need not be invoked if a later contamination by virus is postulated. One possible source of contamination is the food ingested by the larva. It is not known which food the larva was eating prior to being put in the battery jar; but the only artificial diet used in the jar came from the C.A.E.S. Many P. dispar larvae infected with polyhedrosis are reared in the C.A.E.S. laboratory, and it is possible, though not likely, that even with meticulous technique, contamination of food occurred. If the source of virus came from food, however, more than one larva should have been infected. Another possible source of contamination could have been cross comtamination of poly-hedra from the control goup being reared on a different floor. This was cared for by the same experimenter and in it one early polyhedral death occurred. The L.D.$_{50}$ dose in infection with poly-hedra has been found to be, for P. dispar, about 100 micrograms (Aizawa, 1963), a small amount; and it may be possible that infection could occur following ingestion of a single contaminating poly-hedra, accidentally transferred from the control group.

Mortality in the high relative humidity - lettuce diet group. The mortality in this group (35%) was much higher than in the other three. Stressor factors (wet rot of lettuce, mold) contributing to slow development may also have influenced mortality. Also contri-

buting to mortality may be other stressor factors present along with the rotten lettuce. In a warm room, liquified lettuce in a Petri dish along with gypsy moth frass may provide an ideal growing medium for bacteria. If bacterial counts were much higher in these dishes, then one might expect greater morbidity among the larvae if any of the bacteria--even a small percentage of them-- were pathogens of lepidoptera. Under normal conditions a low number of even pathogenic bacteria may be efficiently engulfed by hemocytes; but in the presence of large numbers of other bacteria these pathogens may simply not be phagocytized, or if phagocytized, not killed (Jones 1975; Anderson 1975). The mere presence of these bacteria may be the cause of morbidity and mortality, apart from any physical stressor action on the larvae from high humidity or liquid condensation. Starvation may have contributed to disease. Doane (1970a) suggests that insufficient nutriment may contribute to S. faecalis infection.

Diplococci, presumed most likely S. faecalis, were the organisms most often observed on microscopic examination of these dead larvae. S. faecalis is a known P. dispar pathogen, and may account for many of the deaths. However, various bacilli were also present, and while these were most likely saprophytic organisms some may have been pathogens.

It has also been suggested (Tinsley 1977) that undetected small RNA viruses may have contributed to mortality in this group.

It may be postulated that such viruses, either occurring as con-
taminants or transmitted transovarially in occult form, were act-
ivated by stressors intended to test NPV. No data are available on
possible effects of environmental stressors on such viruses.

The number of factors which may be contributing to morbidity
in this group of larvae demonstrates a major drawback in experiment-
al or observational environmental studies: pinpointing the stressor.
While trying to test the effects of humidity and a diet high in
moisture, complications are encountered which cause unplanned en-
vironmental conditions. Sickness occurring after the appearance of
such complications cannot be attributed definitely to either the
moisture of the food and humidity of the room, or to the excess
condensation and overgrowth of bacteria. Testing each of those
parameters separately without adding additional parameters would
be a difficult and extensive task. The contribution of an environ-
mental factor to the occurrence of disease must often be shown by
an elimination process. In this experiment that was partly attempt-
ed, by having combinations of four factors (high R.H., low R.H.,
artificial diet, lettuce diet) taken two at a time. But as has
been noted, the stress factors for which the experiment was design-
ed engendered additional unintentional conditions. Since these
were not employed in other combinations it is not possible to sort
out the effects of any individual one.

No certain statement can be made about the absolute presence or absence of the virus in many of the larvae. Particularly in the older larvae which contained many fat globules, low concentrations of polyhedral bodies could easily be missed. The visualization of intact tissues (especially tracheal tissue) is good evidence against disease. Glaser (1915) and Vaill et al. (1973) point out that tracheal matrix cells are often the first sites of NPV multiplication, but one may imagine that in one or two cells polyhedra may have been able to form, without actually causing disease. It is not known whether this can happen with viruses of lepidopteran species.

The first instar mortality rate among larvae reared from unwashed eggs, together with the knowledge of the disease level this year in the natural population, is ample evidence that there was a substantial level of NPV disease in the population that produced the eggs for this experiment. Absence of NPV disease in the first instar period in these experimental larvae indicated that the success of the sanitization method of Doane (1969) in decreasing NPV disease has been duplicated in this laboratory. This re-documents what is accepted about the major route of NPV transmission: that it is either via the transovum route, or via debris deposited by the female which holds the egg mass together.

The single later death shows that this was not a virus free population. Viral assays on gypsy moth populations have been com-

plicated by the fact that a low level of viral disease often turns up in the populations, independent of the assays being performed. Some possible latent viral illness may occur secondary to ingestion of polyhedral protein (as occurred for example in the experiments of Longworth, et al. [1968] with foreign polyhedral protein) -- if polyhedra were the form of the virus being assayed. It would be useful to prevent other unexplained occurrences of the disease. If it could be shown that our single viral death occurred because of contamination, then it has been suggested that it might be useful to rear sanitized eggs which had no exposure to materials from other laboratories, in rooms where no P. dispar larvae, and hence no infected P. dispar larvae had been reared previously. It would be helpful, for future bioassays, to know that some random occurrence of polyhedrosis could be avoided simply by using new laboratory rooms.

Effects of Relative Humidity in the Laboratory

High relative humidity did not activate latent polyhedral viral disease in this population, although it contributed to stressor conditions. Yadava's work (1970) gave evidence that even once an infection is started high relative humidity does not necessarily make it worse. Perhaps in this study the relative humidity was not high enough to affect insect susceptibility to the virus. Ninety percent humidity may have a very different effect from the 80%

R.H. tested here. Also, it may be important that these larvae were subjected to high humidity for their entire life spans, and may have adapted to it somehow. Later instars, accustomed to lower relative humidity, may be adversely affected by a sudden increase in ambient humidity. The conclusion that humidity has no effect cannot be stated as a certainty from data given here. In spite of a substantial basic level of NPV disease in the source population, there may have been no occult virus in most of the population tested. The only experiment with P. dispar which is difficult to explain without invoking the existence of a virus in undetectable form (a possible occult virus) is that of Longworth et al. (1968) where native infection developed after ingestion of foreign polyhedral material. Even in that study, or in any study of a stressor known to induce acute infection, even though an occult virus might be suspected, conclusive evidence is not provided of its presence in that form. Even if it were shown that a virus had been transmitted transovarially, and an acute infection induced in the second generation, the infection may not have been latent: it may have been chronic, and perhaps very quiet -- but active (Tanada, 1976).

In this study there was less concern with actual latency, than with the induction of potential disease from a virus in any form not immediately detectable. The best way to determine the presence of virus in this population might be with DNA-annealing tests, attempting to detect the P. dispar nuclear polyhedral virus DNA.

Unfortunately, this work was not within the time scope of this study; but it could be tried with some of the eggs currently under refrigeration. Only by demonstrating the presence of a virus not causing apparent disease can a definite statement be made about the lack of influence of high R.H. in activating the virus.

Effects of High R.H. in the Field.

A discussion of the influence of high R.H. in the field can be much more extensive than one on influences in the laboratory, because of the myriad of parameters involved. (The more active one's imagination, the more the environmental parameters which may be dreamed up.) Disproving an effect of high relative humidity on latent infection in the laboratory does not discount field observations that it seems to influence polyhedrosis of P. dispar and other species. In our laboratory population, individuals were isolated from each other, an artificial situation eliminating many influences which may be present in the field. Crowding has been one influence cited by some authors as a possible stressor in the initiation of an epizootic of wilt disease (e.g. Aruga, 1963; Tanada, 1976; Doane, 1970b). Obviously, isolating individuals eliminates any possible density factor. Perhaps under crowded conditions, high humidity does affect the appearance of viral disease. Whether this could be an influence working at the cellular or biochemical level could, at present, be a matter of speculation only.

Some influences working at the whole-organism level may be postulated. Doane (1970b) suggests that in cool or wet weather larvae just hatched will be slow to disperse, and in dense populations the resultant prolonged intermingling may increase incidence of infection from NPV. Another possible influence of high humidity in natural populations might be some effect on the physical transmission of the disease. High humidity might increase drying time of the remains of "wilted" larvae, making it more likely that the viral material be spread around, by animals (other larvae, other insects, birds) brushing against foliage and transferring viral material to other foliage.

Although it has not been demonstrated for NPV's, relative humidity has been shown to affect persistance of some human viruses (non-occluded ones) in the external environment. Viruses which have lipid components (e.g. influenza viruses and the arboviruses survive best at low relative humidity; while those lacking lipids (e.g., enteroviruses and adenoviruses) seem to survive best at high relative humidity (Buckland, et al., 1962). Humidity may affect persistance of free virions in the forest environment.

All the difficulties encountered in interpretation of stressor action in this study may be found in investigations of stressor activity in viral infections of man and other species. In order to disprove the role of an environmental condition as a stressor, one

must be certain of the presence of the virus. To prove that a particular environmental condition is a stressor, one must be able to separate it from all other environmental influences. It would be useful also to have biochemical information on how an individual stressor might act.

Similarly, in studying latency it is helpful to work with biochemical and serological tools in addition to ecological ones. These seem at present the best way to insure or prove the presence of an unseen virus. As discussed earlier, only when the presence of a virus is known can an environmental factor be disproved as a stressor. Proving a role as a stressor is a little more complicated, involving processes of elimination and deduction, and requiring confidence that extraneous contamination is not occurring. Here again, it may be helpful to use investigational methods not involving the whole organism. If a physical stressor acts at a subcellular level then it may be applied to cell culture. But of course not all stressors can be tested using cell culture. An example in that category is population density.

Biochemical and serological studies also may not always give reliable results and it may be very useful in some instances to know a factor which can act as a stressor in bringing out infection. Mims (1964) has written that the boom in tissue culture (which in some ways made viral studies easier than they were when only the whole animal was available for scrutiny of infectious processes) caused cell-virus interactions to be studied at the expense of the

host-parasite aspect of viral infections. Certainly there is en-
thusiasm for continuing investigation at the level of the whole
organism. In studying the action of stressors this is essential,
as they can, as Selye points out in his discussion of human stress-
ors (1976) have specific and nonspecific effects. Biochemical and
histological studies may help to illuminate the mechanism of action
of some specific stressor effects, but in induction of disease, or
in whatever effect they may have on the organism, specific and non-
specific stressor actions occur simultaneously. The nonspecific
reactions of an organism to a stressor require the presence of the
whole organism in order to be put into motion. In spite of the
complexities involved in study of whole animals and populations,
study at that level is necessary for the understanding of stressor
action.

CONCLUSION

In this study, relative humidity (R.H.) was tested as a stressor in activating nuclear polyhedrosis virus disease in larvae of the gypsy moth <u>Porthetria dispar</u>. Larvae were reared from sanitized eggs in laboratory rooms which had never before housed P. dispar, and which were therefore unlikely to be contaminated with the P. dispar NPV.

R.H. was shown to have no effect on appearance of NPV disease in these larvae. However, in larvae fed a diet high in moisture, it did generate stressor conditions which increased susceptibility of the larvae to other diseases, leading to very high mortality in that group. That mortality may be attributed to <u>S. faecalis</u> or to an undetected virus other than NPV. The possibility was entertained that a small RNA virus activated by the stressor conditions might explain the selective high mortality. It might be worthwhile as a further exercise to attempt to isolate such a virus.

Stressor conditions present in groups other than the high R.H.- high moisture diet one caused retardation of growth and development of larvae. This did not result in high mortality in the other groups; nor did any of the stressors (including malnourishment) produce polyhedrosis.

The absence of NPV disease during the first larval instar in caterpillars hatched from sanitized eggs confirmed the findings of Doane (1969) that nuclear polyhedrosis can be virtually eliminated by effective surface sterilization of eggs. This is consistent

with the currently accepted theory that NPV is not ordinarily transmitted via the transovarial route.

One case of polyhedrosis did occur in a late instar larva. It was concluded that this may be explained by a very long incubation period of a very low inoculum of virus sustained during hatching from an incompletely sanitized egg. The single case of polyhedrosis may also be explained by later contamination from an undetermined source. Possible stressors which may have increased susceptibility of that larva to virus were discussed.

The results illustrated the difficulties encountered in attempting to define stressors and their effects. One major difficulty in disproving with certainty the role of high relative humidity as a stressor was the lack of proof of the presence or absence of an occult virus. Further study in this area should involve biochemical investigation (e.g. using DNA annealing tests) of the presence or absence of the virus in test animals.

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