INFLUENCES OF BENZOYL DERIVATIVES ON THE EMBRYOLOGICAL DEVELOPMENT OF Tribolium confusum. William E. Oakley, Jr. and Gerhard W. Kalmus. East Carolina University, Department of Biology. Greenville, N.C. 27858.

Tribolium confusum, the confused flour beetle, was used as a test model to evaluate the insecticidal potential of benzoyl peroxide, an oxidizing agent used to bleach flour. A known chitin synthesis inhibitor, diflubenzuron, which has a chemical structure similar to benzoyl peroxide, was used as a internal control. Flour was treated with each benzoyl derivative ranging from 0.01 to 100 ppm and progressive developmental stages of T. confusum were exposed to the treated flour for a thirty day period at 27°C and 60% relative humidity. Results indicated that egg laying decreased, the number of larval stages decreased but the time within each larval stage increased, larval weight decreased, and larval and adult mortality rates increased. Measurements of chitin dry weight showed a drop in the overall chitin deposition. Polyacrylamide gel electrophoresis indicated a decrease in total protein concentration in benzoyl treated beetles. Biochemical assays showed a decrease in the quantity of glucose available and a decrease in total protein levels. Histological examinations revealed a weakened and fragile exoskeleton along with many other structural abnormalities. Both treatments had similar effects, but diflubenzuron was more deleterious. Thus, it seems, that benzoyl peroxide could be employed in a dual function, i.e., as a bleaching agent and as an insecticide, thus reducing the necessity of numerous additives in order to achieve pest control in commercially available flour.

INFLUENCES OF BENZOYL DERIVATIVES ON THE EMBRYOLOGICAL DEVELOPMENT OF *Tribolium confusum*

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William Ennis Oakley, Jr.

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by

William E. Oakley, Jr.

APPROVED BY:

SUPERVISOR OF THESIS

Gerhard W. Kalmus, Ph.D.

THESIS COMMITTEE

Clifford B. Knight, Ph.D.

Elizabeth A. McMahan, Ph.D.

Charles A. Singhas, Ph.D.

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

Charles E. Bland, Ph.D.

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette, Ph.D.

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INTRODUCTION

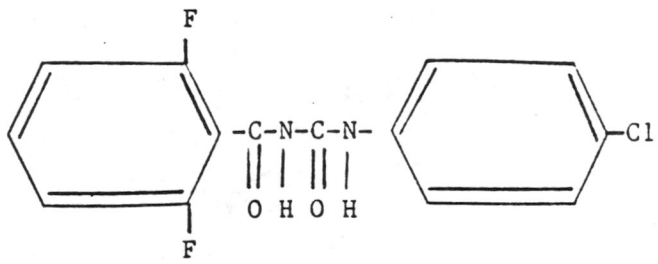
Lack of specificity is a major reason why broad spectrum insecticides produced during the past three decades have often been associated with environmental damage. For this reason, it is becoming more important to develop and use selective insect control agents that are effective, but minimize the hazards to man or the environment. The excessive use of insecticides in preventing insect infestation of various food products has aroused public interest and concern. Many of these chemicals kill a wide variety of insects and are often toxic to animals, including fish, birds, and mammals (Marx, 1977). For example, diazine, a common additive insecticide has been shown to alter respiratory development in fetal monkeys (Rancliff, 1984). Despite increased recent emphasis on biological control with regard to insects, few entomologists believe that the use of chemicals can be totally eliminated. Attempts are therefore being made to develop more specific agents that will effectively control insects without causing further environmental degradation. From an environmental standpoint, it is important that these chemicals be tested in order to determine the degree of possible carcinogenic, teratologic, or growth inhibiting effects (Vincent, 1978).

Many insects that infest grain products contain a moisture dependent exoskeleton which is composed of chitin. The arthropod exoskeleton consists basically of a chitin-protein structure secreted over the body surface by the epidermis, a single layer of cells. The

exoskeleton is usually hardened over most of its surface by the deposition of calcium salts or organic material. These substances are laid down and tanned by an interaction between proteins and quinones. Chitin is continuously spread over body surfaces in varying degrees of thickness and is not restricted to specialized areas. A chemical that inferferes with chitin synthesis and kills insects before they reach reproductive maturity could be used to reduce populations of insect pests that might otherwise be economic threats to farm and forests.

Benzoyl derivatives may be used for insect pest control because they are potent insecticides that act on the larval postapolytic stage and disrupt the molting process, yet are of very low toxicity to mammals. The mode of action of one of the best known benzoyl derivatives, [(1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)-urea; commonly called dimilin], reveals that this compound alters chitin in the insect cuticle.

Figure 1: Diflubenzuron Chemical Structure



The results of diflubenzuron treatment are changes in elasticity and firmness of the endocuticle. The biosynthesis of chitinous skeletal structures is a promising molecular target for pesticide action

because chitin is limited to distinct layers within the insect. Diflubenzuron acts on larvae of different orders causing in some cases, strong larvicidal activity. Its oxidizing action is manifested through dehydration of the larvae. It is this drying action that also increases the shelf-life of various grain products (Mulder and Gijswijt, 1973).

The present study addresses the effects on insect development by two oxidizing agents, benzoyl peroxide and diflubenzuron, both of which are often added to certain grain products to increase their shelf-life. Benzoyl peroxide was chosen from a list of many possible benzoyl derivative candidates, as the one derivative to be tested. The beetle, Tribolium confusum, was used as the test model. The objectives were: 1) to observe the effects of benzoyl peroxide on growth and development of T. confusum; 2) to compare these results with those of a known chitin inhibitor, diflubenzuron; and 3) to evaluate these benzoyl derivatives, which are presumed not to be harmful to man, as insect control agents.

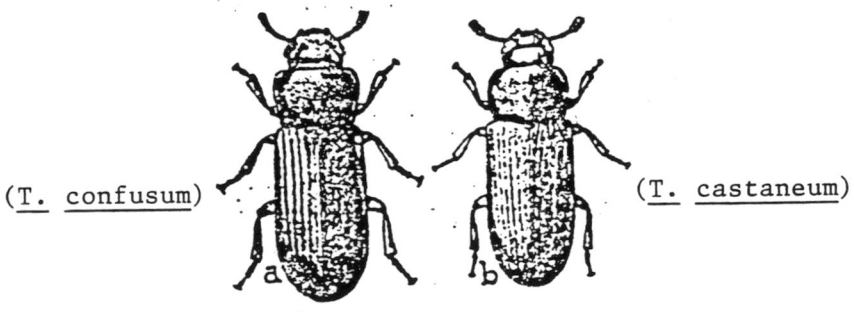
REVIEW OF LITERATURE

Tribolium confusum, the flour beetle, possesses characteristics which commend it as a good research model. It is cosmopolitan and easily obtainable and has a moderately short life-cycle. This beetle is hardy and requires no elaborate equipment for its maintenance. Adults and immature forms are readily recoverable and their entire life-cycle is spent in pulverized grains, such as flour. Despite these attractive characteristics, it is only recently that Tribolium has been used by biologists interested in theoretical problems. The economic zoologists have been aware of the species for many years and has provided plenty of data on the general ecology of the species. Babcock (1912), an early worker using Tribolium, studied the problem of metabolic water, finding that this insect maintained a tissue water level about 40% greater in relation to the 10% moisture content of its environment. Davey (1917, 1919), reported that the longevity of these beetles could be prolonged if mass cultures received a small dosage of X-rays. The radiation exposure seemed to slow the aging process in these cultures. Probably, most credit in using Tribolium as an experimental organism, belongs to Chapman who in 1918 reported on its nutritional requirements, and in 1928 on its suitability for population studies. In light of these early studies, the following general topics about Tribolium confusum will be reviewed including, taxonomy and history, the food relationships of the flour beetle, life-cycle and environmental factors, and productivity (fecundity and fertility).

Taxonomy and History

The genus Tribolium is a member of the Order Coleoptera, Family Tenebrionidae, and sub-family Ulominae. The Tenebrionids are commonly referred to as the "Darkling beetles" and include such forms as certain fungus beetles, the pinacate bugs, and common meal worms, (Tenebrio molitor). There are several other species of Tribolium besides confusum. The other common American form is Tribolium ferrugineum. Good (1933) states that the latter species is probably more properly referred to as Tribolium castaneum. These two American flour beetles seem to be remarkably similar in general structure and function. There are several criteria used by taxonomists in distinguishing the two from each other. T. castaneum possesses a distinct three-pointed antennal club while T. confusum has a gradually enlarging club. Good (1933) points out the fact that the eyes of T. confusum, when viewed from the ventral surface, are smaller than those of the other species and T. confusum has a more southern range of distribution than T. castaneum (Jaques, 1951).

Figure 2: Morphology of T. confusum vs T. castaneum



At the present time, the genus Tribolium is very widely distributed over the world. It is largely disseminated in grains transported by commerce. The exact origin of the grain dwelling habitat is not known, but a probable interpretation is advanced by Good (1933): "Almost without exception, the beetles of the sub-family Ulominae, of which Tribolium is a member, occur either as pests of stored products or else under the bark of trees and in rotting logs. It seems evident that all members of this group originally lived in the latter habitat and have recently adopted the flour feeding habit. Two species of Tribolium, T. madens and T. indicum, are found almost exclusively under bark or in rotting logs and the two flour pests, T. confusum and T. castaneum, are themselves occasionally found there."

T. confusum was first described unknowingly by Mulsant (1854), who, in attempting to improve upon the account of the already known T. ferrugineum, published a description of confusum. However, since Mulsant thought he was simply redescribing T. castaneum, his reference to confusum has been neglected. The credit of the description has been ascribed to du Val (1868), who recognized T. confusum as a distinct species and published his account.

Nutrition

T. confusum lives in almost any kind of flour, cracked grain, breakfast food or meal. A list of specific foods in which these beetles are found has been compiled by Chittenden (1896, 1897) and

includes whole-wheat flour, bleached and unbleached white flour, bran, rice flour, rye flour, corn meal, barley flour, and oat meal. Good (1933) also reports the beetles living in chocolate, spices (red pepper), various kinds of nuts, and sometimes feeding on specimens in insect collections. Chittenden (1897) has found Tribolium in snuff, orris root, baking powder, ginger, slippery elm, peas and beans. The beetles are unable to feed on whole grains, as pointed out by Chapman (1931), because their mouth parts are not adapted for attacking large, hard pieces of food. Typically, the entire life-cycle of a Tribolium is passed within its original environment.

Chapman (1918) has studied the relative susceptibility of wheat flour to the invasion of Tribolium in order to determine if there is any 'preference' exhibited by adult beetles for specific flours. This investigator divided an experimental jar into five equal portions, each containing a different kind of wheat flour, which varied from finely ground flour (first middlings) to bran. These flours were in continuity with each other so that a beetle could pass from one type to another. Adults were introduced into the center of the jar and were allowed to migrate to any of the five types of cereals. After varying lengths of time, flours were examined and a census made of their beetle populations. Chapman found that the bran contained essentially twice as many beetles as did the other media and concluded that this data indicated a preference selection on the part of Tribolium. In repeating the experiment with larvae, no evidence of such a 'preference' was found. Observations were also made with rice flour, rye flour, barley flour, and corn flour, and theresults of these ex-

periments indicated that the beetles were reacting, not necessarily to the richest food, but to the most coarsely ground medium. Chapman ingeniously tested this hypothesis further by running a varying series of sawdusts differentially ground and again corroborated the fact that the beetles reacted positively to the coarse material. However, after the experiments had run a longer time, it was observed that all of the flours, whether coarse or fine, were equally populated. This was because the beetles eventually honeycombed the finely ground flour with tunnels and were then able to move freely through it.

Chapman (1924) reared Tribolium on various media and concluded that microorganisms did not play an important part in their nutrition, nutritional requirements for growth were less exacting than those for maturity or metamorphosis, and wheat germ most closely satisfied the physiological requirements of Tribolium. He also found that Vitamin B was necessary for their development. His investigations showed that the wheat embryo was a rich source of Vitamin B. Conclusions drawn from these experiments indicated that flours represent a well-balanced diet and that Tribolium is typically able to live in them with its complete nutritional requirements well satisfied.

It is interesting to briefly mention certain experiments conducted by Holdaway (1933) dealing with the effect of starvation on sex ratio. This investigator subjected first instar larvae to different degrees of starvation as follows: the first group served as controls and were not starved; the second group was starved for one day; the third group was starved for two days; and the fourth group was starved for three days. These experimental groups were incubated to pupae when

sex was determined. Larvae starved for one day had a higher proportion of males compared to the controls. This higher proportion was not statistically significant. However, the groups starved two and three days had a statistically significant increase in the number of females when compared with the control group. Holdaway said the difference in sex ratios can not be explained on the basis of a differential mortality between sexes, but starvation is the critical factor.

Metamorphosis and Life-cycle

Tribolium confusum, a holometabolous insect has in its life-cycle; egg, larval, pupal, and adult stages. Eggs are ovoidal, but usually appear irregular because they are often surfaced with flour particles. Brindley (1930) reported the following egg measurements: average width 0.4 mm, and average length 0.63 mm, with a standard deviation of 0.02 and 0.04 mm, respectively. Chapman (1918) said that contour and general appearance of the eggs varies with the medium in which they are found. Eggs in finely milled wheat flour appear smaller than those found in coarse cereals. Stanley (1932) determined the average moisture content of 30,000 eggs and found it to be 44.9%.

The eggs hatch into small, white larvae with an average weight of 0.028 mg. The molting behavior of the larvae may be described in the words of Chapman (1918), "For a short time before each molting, the larva is inactive and the body is large in proportion to the head. The skin splits dorsally over the head and thorax, and the larva emerges. It is at first white, like the larva of the first instar, but after

twenty-four hours it takes on a yellowish color. Immediately after molting, when the larva has expanded as a result of being freed from the old skin, it has often been observed to remain quiet for a time."

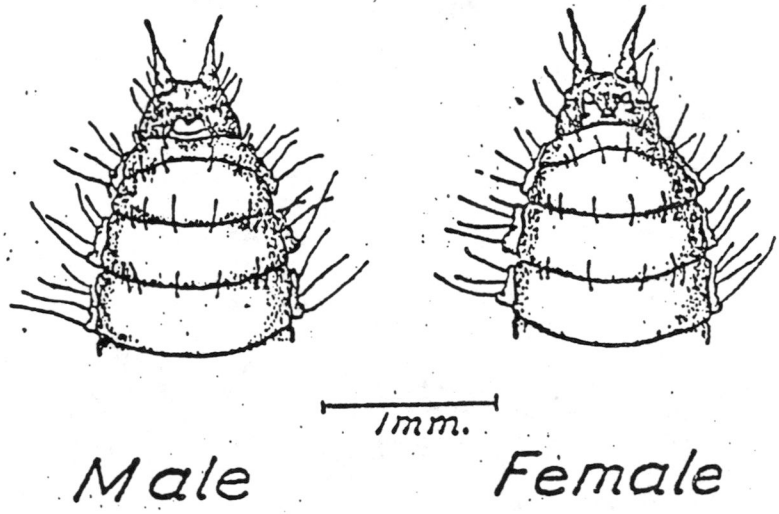
Chapman (1918) and Brindley (1930) noted that there were six larval instars in development. These investigators measured the larval head width during the different instars and used this as a criterion of development, i.e., a certain head width indicated a specific larval stage. Good (1933) studied the number of instars by counting the number of exuviae (larval skins) deposited in the flour after each larval molt. This author concluded: "...the writer has determined that there is no fixed number of larval molts, but that the number ranges from 6 to 11 or more and is normally 6 to 7. This variation is due both to external conditions, such as food, temperature, and humidity, and to individual characteristics entirely apart from external influences."

The larvae gradually increase in size with every molt: Brindley (1930), said the fourth instar larvae, for example, had an average length of 3.23 mm, a head breadth of 0.4 mm, and a weight of 0.332 mg. The last instar larvae are 6.0 mm long, 0.69 mm broad across the head, and weigh 2.4 mg. As the time for pupation approaches, last instar larvae become more quiescent and contracted.

The pupae of Tribolium are naked, with the occasional exception of a slight abdominal movement, they are inactive. They are pale yellow when first formed but turn darker yellow with age, becoming brown at the time of emergence. Brindley (1930) reports pupae had a mean length of 3.46 mm, and a width of 1.12 mm. There was a tendency for female pupae to be longer than males. A question of great practical

importance for investigators is the method of determining the sex of beetles. As far as is known, the only reliable external sexual characteristics for any stage is found in the pupal stage. When the ventral posterior ends of the male and female pupae are examined under low magnification, the sexual distinction is obvious. On the terminal segment, the female has a pair of small appendages which are reduced to indistinct elevations in the male.

Figure 3: Sexual Distinction Between Male and Female Pupae



Chapman (1924) pointed out the duration of the last larval instar is more influenced by ecological changes more than any other developmental stage. Average length of time required for the completion of a Tribolium life-cycle under Brindley's (1930) conditions of temperature, humidity, and food was 30 days. Brindley's (1930) data, demonstrated that for a particular set of conditions, and a presumably in-bred stock, the variation in the length of the life-cycle is surprisingly small for observations based on 100 individuals. It would be incorrect to say that 30 days represents the usual length of the cycle. Good

(1933) showed the length of the life-cycle varied according to the food the larvae are fed. At 27° C, the larval period lasts 31 days when the forms had been reared on middlings as compared to 89 days when the larvae had been raised on white flour. The latter observation differs from Park's (1932) experiments with white flour at a temperature of 28° C, and a relative humidity of approximately 50%. An entire life-cycle from egg to adult under these conditions was about 36 days. The length of the life-cycle increases with lower temperatures. Good (1933) reported that Tribolium adults spent the winter in unheated Maryland flour mills in a semi-dormant condition and resumed breeding when spring approached. The larvae and pupae do not seem to withstand low temperatures as well as the adults. Chapman (1931) reported that Tribolium adults died in a few weeks if subjected to a temperature as low as 7° C, indicating their inability to assume a true dormancy. On the basis of the experiments of Chapman and Baird (1933), the relation of temperature to the length of the life-cycle can be summarized as follows: at 32° C, the development from egg to adult took about 25 days; at 27° C, 35 days; and, at 22° C, 83 days. These findings were obtained under constant conditions of humidity (75%) with whole wheat flour.

The length of the life-cycle of Tribolium confusum must be expressed in terms of the environmental conditions that transpire at the time of the observation. Although no data are available, it is more probable that the duration of metamorphosis varies according to the genetic composition of the beetles as well as with the surrounding ecological conditions.

Holdaway (1933) has reported at length upon the effects of atmospheric moisture on Tribolium. He dealt with the following aspects of the subject: 1) the effect of humidity on the size of adult populations; 2) the rate of population growth as related to humidity; 3) the effect of change of humidity on population equilibrium; and 4) the relation of humidity to the rate of development and the viability of the various metamorphic stages, e.g., physiological effects of humidity in affecting entire populations. Holdaway (1932) found adult Tribolium increased in numbers as the humidity increased from 25% to 75%. Above 75% relative humidity, the number of adults declined as fungus appeared in the flour. He noted that the adult populations kept at 75% relative humidity had a faster growth rate than those at lower humidities. By taking an asymptotic population of adult beetles characteristic of a 25% humidity environment (9.5 beetles per gram of flour) and subjecting this same group to a humidity of 75%, the population increased to a concentration typical for a 75% humidity (15 beetles per gram of flour). This indicated that the effects of humidity on the life-cycle are probably not irreversible in nature. Holdaway (1932) found that, more eggs hatched in low humidities than in high, but the larvae showed a greater survival in the high humidity environments with an optimum at 75%. The pupae had coefficients of survival similar to those of the eggs since more pupae failed to develop as the surrounding atmospheric moisture increased. The effect of humidity on rate of metamorphosis showed that eggs and pupal stages were little influenced by humidity conditions, but larvae developed more rapidly as humidity in-

creased from 25% to 75%. Holdaway (1932) investigated the influence of 25%, 50%, and 75% relative humidity on oviposition and found that females in 75% humidity produced significantly more eggs than those in 25%. When the females of the 50% environment were compared with those in the 75% environment, there was a suggestion (1.9 times the standard error) that the former were ovipositing more rapidly.

As an elaboration of the life-cycle of Tribolium confusum it might be well to describe briefly some of the facts known as to the growth of entire populations of these forms. Chapman (1928) has experimentally approached this problem with interesting results. He set up six environments of whole-wheat flour which increased in size geometrically. The environments consisted of 4, 8, 16, 32, 64, and 128 gm of flour. Adult Tribolium were introduced into these environments to provide initially one beetle for each 2 gm of flour (4 gm culture contained 1 pair, 16 gm culture 4 pairs, 32 gm culture 8 pairs, 64 gm culture 16 pairs, and 128 gm culture 32 pairs). Counts of eggs, larvae, pupae, and adults were made in order to see how the total population was increasing relative to environment size (gm of flour). Chapman (1928) found that after approximately 100 days, all populations reached an equilibrium when measured in terms of beetles per gm of flour. In other words, the culture of 128 gm of flour with its initial population of 32 pairs of beetles, although much larger after 100 days in terms of total number of individuals, contained essentially the same number per gm of flour as did, for example, the 4 gm-1 pair unit. Chapman calculated this equilibrium point attained by Tribolium under these specific conditions of temperature, humidity, and kind of flour, to be 43.97

± 2.88 individuals per gm of flour. Chapman considered this equilibrium to be the result of the interplay between the capacity of the species to reproduce (biotic potential) and the resistance of the environment. He concluded that cannibalism was a most important factor in this resistance, since, although thousands of eggs may be present in the environment at any one time, only some of them escape being eaten. Egg consumption was directly proportional to the population concentration and maintained the equilibrium of the total number of individuals.

Productivity

Of obvious importance to investigators is the question of productivity. This can be interpreted as dealing with both oviposition, or fecundity, and fertility, or percentage of egg hatch. It is only recently that data have been reported on these considerations and much still remains to be done. Good (1933) reported a series of interesting observations on oviposition which are valuable since they record the number of eggs produced by females during the entire period of their egg-laying life. This period may last as long as 14 months, the average being approximately 9 months. During this time, a female Tribolium normally lays 400 to 500 eggs, although records of nearly a thousand eggs are not unknown. With regard to egg fertility, about 90% of these eggs hatch. Brindley (1930) obtained oviposition data for 10 newly emerged Tribolium pairs and found that these young females oviposited at a much

higher rate during this limited period than did the forms used by Good (1933), who obtained longtime records. Using Pearl's (1922) rate per female per day method of measuring fecundity, Brindley (1930) reported (for this 10 day period) an average of about 11 eggs per female per day. These rates are higher than those of Chapman (1918) or Park (1932), who also worked with young female beetles over a 10 month period of observation. One reason for the discrepancy is that Brindley (1930) removed his adult beetles daily and introduced them into fresh flour, eliminating consumption of eggs by adults. In addition, other factors, both generic and ecological, are quite conceivably operating in this case. Temperature affects oviposition because according to Stanley (1932) at 22° C, the average oviposition rate per female per day was 1.9 eggs, at 27° C, 6.24 eggs, and, at 32° C, 10.73 eggs. These are all statistically different and clearly indicate the relation of moderate temperature variations to fecundity. The rates presented here are not therefore absolute fecundity rates but simply a measurement of the eggs remaining in the population after some have been consumed. Park (1932), in studying population problems, had occasion to frequently repeat a standard experiment in which either one pair of young adult Tribolium or else a single fecundated female spent 11 days in 32 gm of flour at 28° C. Egg counts were made at the end of that time. The emphasis here was placed upon the study of natural population in which egg-eating is an important function. Park (1932) used flour in his experiments while Chapman (1918) and Brindley (1930) used whole-wheat flour. Good (1933) has shown that the kind of flour influ-

ences egg-laying. At 27° C, in whole-wheat flour, he found the average egg rate per female per day was 2.43 eggs; in bran, 1.26 eggs; in oatmeal, 1.04 eggs; and, in white flour, 0.58 eggs. There is in all probability, no real discrepancy between these data because experimental conditions and procedures have been different with various ultimate aims in mind. About oviposition, Good (1933) says: "The number of eggs laid per day is not large. In no case were more than 13 viable eggs laid in one day by a single female, and the average was only 2 or 3 per day. Under optimum conditions Brindley (1930) records 18 eggs in one day and a much higher daily average than is indicated here."

It is a common observation that one female may have a high oviposition rate which persists throughout a considerable portion of that individual's life, while another female under similar conditions, has a consistently lower oviposition rate. Facts such as these seem to indicate that the genetic constitutions of the beetle in question is an important factor in fecundity as well as are ecological influences. With respect to egg fertility, Park (1933) found that about 90% of the eggs produced larvae providing they were laid by a female who had experienced more than a single initial copulation. Park (1933) also reported the oviposition rate of young virgin Tribolium females 20 times lower than that of non-virgin individuals.

An interesting, but anticipated, feature of Tribolium oviposition is that it is affected by certain environmental influences, such as kind of flour, humidity (Holdaway, 1932), temperature, conditioning of the flour by beetles living in it (Park, 1934), as well as population density relationships.

Chitin Metamorphosis and Ecdysis

Chitin is an important constituent of the exoskeleton of insects since about a third of the dry weight of the entire cuticle is composed of this polysaccharide (Wigglesworth, 1953). An up-to-date definition of the insect exoskeleton might be that it consists basically of a chitin-protein structure, secreted over the whole body surface by an epidermis consisting of a single layer of cells. Chitin is built up of long unbranched chains composed of N-acetyl-D-glucosamine residues, joined in 1-4-B-linkages; it is not found in the pure state in the cuticle, but always in association with protein. The cuticle is usually stiffened and hardened over most of the surface of the body by the deposition of calcium salts or organic material laid down and involving tanning due to interaction between proteins and quinones. The postulated scheme for chitin biosynthesis is shown in Figure 4.

As insects develop from immature larvae to adults, they undergo several molts, forming new cuticles and shedding their old ones. Chitin formation begins at the end of each larval instar and continues through pupation and the first few days of adult life (Candy and Kilby, 1962). The structure of the chitinous exoskeleton is composed of four main strata, which are easily recognizable structurally. They are usually termed the epicuticle, the pigmented layer, the calcified layer, and the uncalcified layer. A difference between the layers is that the pigmented, calcified, and uncalcified layers contain chitin but this substance is absent from the epicuticle. Hence, it is often convenient to

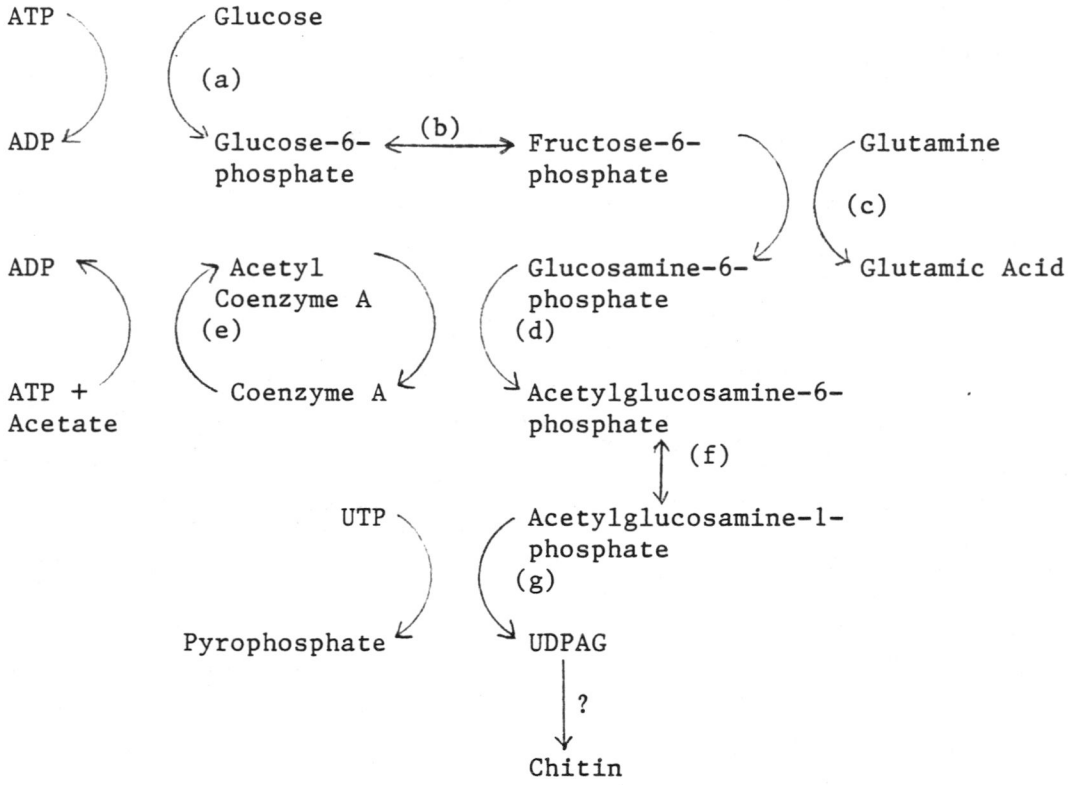
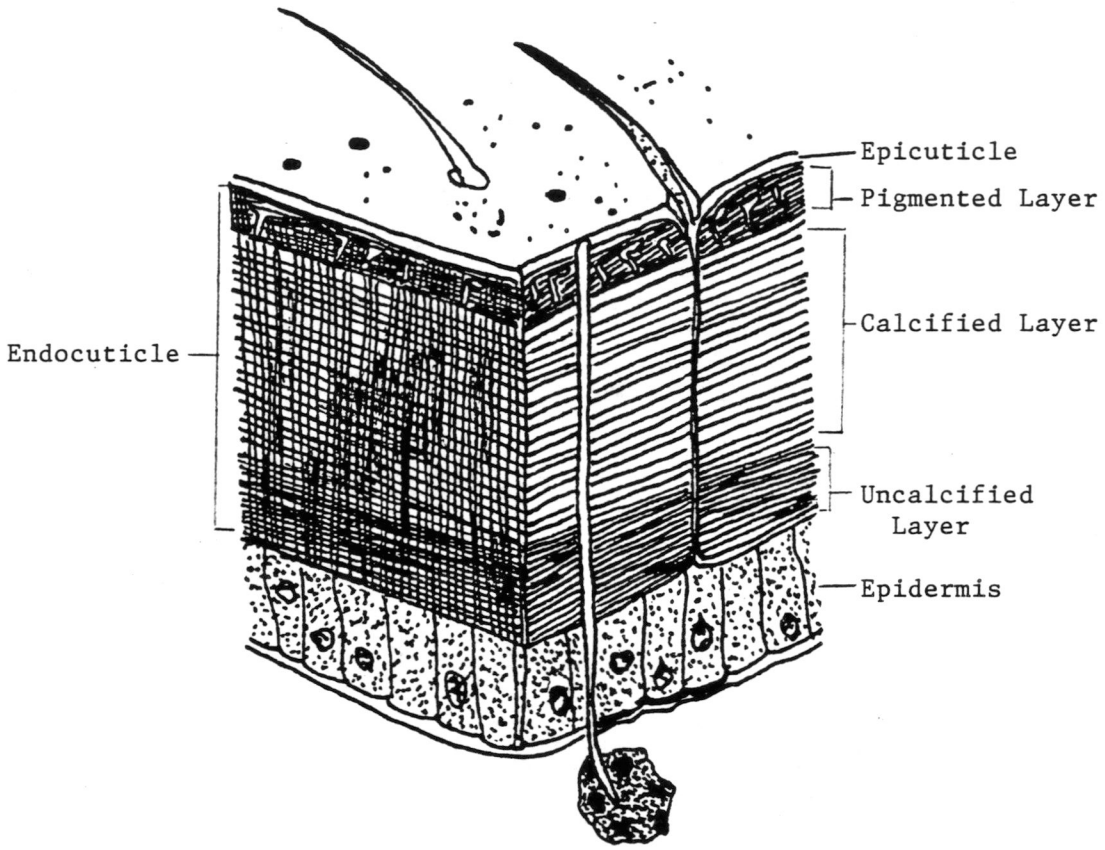


Figure 4: Postulated Scheme For Chitin Biosynthesis
a, hexokinase; b, phosphohexose isomerase; c, glutamine transaminase; d, phosphoglucosamine transacetylase; e, acetyl coenzyme-A synthetase; f, phosphoacetylglucosamine mutase; and g, UDPAG pyrophosphorylase

speak of the chitin-containing layers collectively as the endocuticle. These layers, with the possible exception of the epicuticle, are laid down successively during the development of the cuticle. The new cuticle is formed before the overlying cuticle is shed. But not all the layers of the cuticle are formed before ecdysis. The uncalcified layer appears afterward, and is therefore distinguished as postecdysial in origin, in contrast to the remainder of the cuticle.

Figure 5: Diagram of Cuticle Layers



Prior to ecdysis the cells of the epidermis become greatly elongated. The old dissolving cuticle is invaded by nuclei which are believed to play a part in the softening process. A molting fluid is found between the old cuticle and the epidermis. The two essential cuticular changes involved in molting are: 1) the softening or dissolution of the inner layers of the old cuticle, which liberates it from the epidermis; and, 2) the growth of the new cuticle. It is the presence of a thin and elastic new cuticle that allows an expansion of the body as growth in size of the animal takes place. Though dissolution of

the old cuticle is restricted to the inner layer over most of the body surface, there are certain well-defined areas in which the whole thickness is softened. This facilitates fracture and escape of the animal from its old exoskeleton.

Growth in size of the animal results from the copious intake of water after molting, with consequent increase in the volume of the body fluid. As a result, the new cuticle becomes stretched. Because permeability of the new cuticle cannot be limited by calcification if it is to remain elastic, probably one of the functions of the lipid-containing epicuticle is to render the integument relatively impermeable to water during the critical period of expansion.

Although the old cuticle cannot be shed until a new one has been formed to replace it, shedding does not invariably follow the growth of a new cuticle. The epidermis does not retract from the old cuticle, but becomes reorganized as two layers. The outer one adheres to the old cuticle and is shed with it; the inner gives rise to the new cuticle.

Diflubenzuron

Diflubenzuron was discovered by scientists at Philips-Duphar, B.V., in the Netherlands and is being developed by the Thompson-Hayward Chemical Co. for market in the U.S., under the trade name "dimilin". Many investigators are enthusiastic about the potential use of this compound for controlling a wide variety of insect pests. It is

relatively specific, having only a low, short-term toxicity for fish, birds, and mammals (Marx, 1977).

Diflubenzuron is a benzoylphenyl urea. The current consensus is that diflubenzuron acts by inhibiting chitin synthetase, the final enzyme in the pathway by which chitin is synthesized from glucose. Indirect evidence supporting this hypothesis, comes from the work of the Philips-Duphar group. They compared the action of diflubenzuron with that of polyoxin D, a known inhibitor of chitin synthetase, and found that the two agents produced indentical effects on insect cuticles (Marx, 1977).

Because all insects need to synthesize chitin during development, diflubenzuron can control a wide variety of insect pests, according to Ferrell of Thompson-Hayward (Marx, 1977). Ferrell said that it is especially effective against most "worms", (i.e., caterpillars and larvae that feed on foilage and grain products). In addition, diflubenzuron can control certain beetles, flies, mosquitoes, gnats, mites, and weevils. These insects feed in such a way that they ingest the compound or they absorb it efficiently either from the digestive tract or through the cuticle.

Most studies aimed at determining whether diflubenzuron has adverse environmental effects have indicated that the compound is benign, at least compared with some agents used in the past. Booth (1983), has conducted many laboratory and field studies on the environmental effects of diflubenzuron and says that it is the safest insecticide that he has ever seen. Field trials with diflubenzuron conducted in Pennsyl-

vania during the past three years, showed that the agent reduced the population of beetle larvae by 95% (Ungar, 1986).

McGregor and Kramer (1975) suspecting that diflubenzuron might be useful as a protectant for stored grain, applied it on wheat and corn, and evaluated the activity against seven species of Coleoptera. These included the rice weevil, granary weevil, maize weevil, lessor grain borer, confused flour beetle, and the cigarette beetle. It was demonstrated that diflubenzuron was highly active as an inhibitor of development in all the Coleopteran insects tested. Low doses of diflubenzuron protected wheat and corn for several months against progeny from both internal and external grain feeding insects when the chemical was homogeneously mixed with whole kernals. Since treated grain showed no appreciable damage from infestation, any progeny most likely died in the embryonic or early larval stages. A dose of one ppm on corn was sufficient to control all test species. Diflubenzuron also caused mortality among young adults and affected fecundity of all species. The production of progeny by the confused flour beetle was reduced 98% or more when the insects were exposed to wheat treated with 10 ppm diflubenzuron.

Grosscurt (1977) demonstrated the larvicidal and ovicidal activities of diflubenzuron. On larvae it acted mainly as a stomach poison, yet it sometimes exhibited important contact activity. Though all instars can be controlled, older instars were generally less susceptible than younger ones. Histological inspections of T. confusum larvae revealed that after ending exposure to the compound, distortions in new-

ly deposited cuticular layers decreased gradually. Ovicidal effects resulted from direct contact of diflubenzuron with eggs or from contamination of females by contact or feeding. Electron microscopic observations of embryos of T. confusum, contaminated via the female, also showed disturbed cuticle formation, suggesting a similar activity of the compound in larvae and in eggs.

Ascher and Nemny (1976) investigated the effect of diflubenzuron as a residue on glass or applied topically to T. confusum larvae. Diflubenzuron was active as a residue on glass against 1 and 2 mg larvae. By topical application, diflubenzuron had an ED_{50} for cumulative percentage mortality up to the adult stage of 0.04 and 0.66 micrograms per larva for 1 and 2 mg larvae, respectively. The data indicated that diflubenzuron has contact activity as well as the known stomach poison action.

Ishaaya and Ascher (1977) investigated the effect of diflubenzuron on growth and carbohydrate hydrolases of T. confusum. They found that the potency of diflubenzuron was much greater at inhibiting growth and development of 5th instar larvae of T. confusum than of 1st instar larvae as expressed by death at the apolytic stage and retardation of larval development. A dose-dependent decrease in the activity of trehalase, invertase, and amylase, was obtained with the increase in diflubenzuron concentration. At 5 ppm dietary concentration, a reduction of 17% and 27% in invertase and trehalase activity, respectively, was obtained in 5th instar larvae fed for 3 days on treated diet. The amylase activity was affected to a lesser extent. The observed disturbances

of trehalase activity might hamper the supply of glucose needed for chitin build-up and those of invertase and amylase might affect feeding.

Deul and co-workers (1976) investigated the inhibition of chitin synthesis by diflubenzuron. They explained the biochemical mode of action of diflubenzuron in reference to its insecticidal effects. Virtually complete chitin inhibition was demonstrated 15 minutes after the application of diflubenzuron. Chitinase activity was not effected and the insecticidal effect was explained as an inhibition of chitin synthesis and not as an activation of chitin degradation. They found that there was no accumulation of N-acetylglucosamine following treatment.

Benzoyl Peroxide

Benzoyl peroxide, $(C_6H_2CO)_2O_2$, has long been used as a drying agent for skin disorders in many pharmaceutical drugs, but only within the past two decades has it been utilized as an oxidizing agent in bleaching the yellow color of freshly milled flour (Potter, 1973). This oxidizing technique protects the flour against harmful moisture build-up, which simultaneously increases the flour's shelf-life.

As an antiparasitic agent, benzoyl peroxide was shown useful for treatment of lice and scabies (Parrish, !978). In his study, benzoyl peroxide was useful for treating the larval and adult forms of both species of parasite, but not in destroying their fertile eggs. The main effect of benzoyl peroxide on larval and adult forms was the dehydra-

tion of tissue. This drying action led to the demise of the infesting organism.

Wyatt (1967) suggested that the drying action of benzoyl peroxide could result in a dehydrated condition for infesting flour beetles. By altering the insects body fluid levels, one could prevent the growth and development of maturing larvae, which are very moisture dependent. The molting process, which advances the beetle larvae to more mature stages, is halted when dehydration becomes evident.

In the dosages used today, benzoyl peroxide has been shown to be nonharmful to mammals. Sharratt and co-workers (1964) fed groups of rats and mice on daily diets containing benzoyl peroxide at 10, 100, and 1000 times the average human daily intake. The average human daily intake of benzoyl peroxide is estimated at 2.5 ppm. No significant differences were found between control and experimental groups regarding mortality rates, nor was there any detectable carcinogenic hazard noted.

MATERIALS AND METHODS

All insects obtained were from cultures maintained in our lab and had no prior history of exposure to either benzoyl derivative. All experiments were conducted at 27±2°C and 60±5% relative humidity. These conditions optimized a thirty day life-cycle for T. confusum as determined by a preliminary study which traced the life-cycle from egg to adult under these conditions.

Flour from the same lot was used in all experiments and was expressed quantitatively in terms of weight and surface exposure. All experiments were maintained by as constant external conditions as possible so that a controlled and reproducible environment was established.

I. Preliminary Studies

A preliminary study was conducted to establish the proper conditions under which T. confusum could mature from egg to adult within a reasonable period of time. A second study was then undertaken to establish which developmental stage appeared to be most affected by a series of concentrations of each benzoyl derivative.

A. Thirty Day Life-cycle (Temperature and Relative Humidity)

The culture of T. confusum was maintained in a covered 8" x 11" dissecting pan with 20 gm of flour. Using a 32 mesh and 60 mesh seive combination, (32 on top, 60 on bottom), the entire culture was sifted; thus, separating the adults and larval forms in the 32 mesh seive from

the eggs and exoskeletal remains in the 60 mesh seive. Adult and larval forms were returned to a new stock culture.

Using a dissecting microscope (with the 4X magnification in place) and a small (eyelash paint) brush, the sticky eggs were extracted and 5 of them were placed into each well of a 6 well tissue culture plate. This plate was placed in an incubator which allowed for an adjustment of temperature and relative humidity. Based on the studies of Park (1933), it was determined that an approximate 30 day life-cycle could be established with a temperature of 27°C and 60% relative humidity, provided sufficient flour was present for the beetles to eat. On a daily basis both the temperature and relative humidity were recorded. Daily measurements were also recorded on the number of days it took for the eggs to hatch. Once the first instar larvae appeared, a ½ gm of fresh flour was added to each well in order to feed the developing forms. With a dissecting microscope and a 5 place decimal scale, the body length, head capsule width, and total body weight of each larvae was measured on a daily basis. These three parameters were used to separate each larval instar based on Park's classifications. Simultaneously we monitored the number of days it took each larval instar to mature to its next stage of development. Measurements were continued through pupation until adult forms were observed.

B. Determination of the Larval Stage Most Susceptible to Treatment

In order to determine which developmental stage was most significantly affected by exposure to benzoyl peroxide or diflubenzuron, it was felt a biochemical approach was appropriate. Utilizing poly-

acrylamide gel electrophoresis, the protein changes were examined which resulted following 30 day exposure to both derivatives.

A 1.5 mm thick, 7.5% gel was ran at 35 mAmps for 4-5 h. Under constant current conditions, the voltage gradually increased during the run. Gels were stained with 0.1% Coomassie blue in fixative (40% MeOH, 10% HOAc), for 30 min. then destained with 40% MeOH/10% HOAc to remove background (usually 1-3 h). Gels were stored in destaining solution. After storage, gels were restained with a fluorescent compound, Flu-Vis, which combined with the protein bands in the gels. The bands were read in a densitometer for quantitation by utilizing a measured peak area to give the readings for percent total protein present in each band. See appendices for gel preparation, sample preparation, gel casting, and sample loading protocals.

II. Fecundity

A. Number of Eggs Laid Following Exposure

Thirty female adult beetles were exposed for a 30 day period to both derivatives and then removed from treatment. The beetles were stored in three 8" x 5" x 3" Tupperware containers with 10 gm of stock flour in each; ten females per container. Group one served as control, and remained untreated. Group two contained flour treated with 10 ppm benzoyl peroxide. Group three contained flour treated with 10 ppm diflubenzuron. All containers were maintained at 27±2°C and 60±5% relative humidity for a 30 day period. Following exposure, the beetles were returned to secondary containers which contained 10 gm untreated

flour and for the next 3 day period, eggs were counted utilizing a dissecting microscope and a small brush. Eggs were removed to empty 9-cm petri dishes and placed in an incubator in order to observe the number of viable eggs present.

B. Number of Viable Eggs Following Dipping

Eggs of T. confusum were obtained as follows; fertilized females kept at 27±2°C and 60±5% relative humidity were allowed to lay eggs on filter paper in 9-cm petri dishes. From the onset of the first oviposition, the filter papers were changed every morning. Oviposition took place only during the night, but eggs that were used for experiments on the same day as the morning collection, were designated as 0-1 day-old. Eggs were carefully removed with a fine brush, examined under the microscope, and any defective egg, shell debris, etc., was removed. The eggs were divided into groups of 10 and placed on small 8-cm circular pieces of clean filter paper. For each benzoyl derivative concentration (0.01 ppm, 0.1 ppm, 1.0 ppm, 10 ppm, and 100 ppm), there were 3 groups of 10 eggs. The small filter paper pieces with 10 eggs were picked up by the free margins with forceps and dipped for one second into freshly prepared aqueous dilutions of benzoyl peroxide and diflubenzuron. The pieces of paper were then placed in 9-cm petri dish covers and placed in an incubator at 27±2°C and 60±5% relative humidity. Eclosion started on the 3rd to 4th day. Emerging larvae were removed to a secondary culture for further developmental observations. The numbers of unhatched eggs and empty egg shells were counted when hatching in the controls was complete; data were confirmed a day later (Ascher et. al., 1979).

III. Environmental Parameters on Survival

A. Changes in Larval Weight, Pupation Rates, and Mortality Rates

Larvae were allowed to mature to the 5th instar stage under the conditions established in the preliminary studies; under normal conditions this took approximately 12 days. These larvae were exposed to a series of concentrations (0.01 ppm, 0.1 ppm, 1.0 ppm, 10 ppm, and 100 ppm) of each benzoyl derivative for a 12 day period following eclosion. There were 30 larvae per concentration, e.g., 30 larvae for the 0.01 ppm benzoyl peroxide treated flour, 30 larvae for the 0.01 ppm diflubenzuron treated flour, etc. The larvae were contained in 9-cm petri dishes with 3 gm of treated flour. Following 12 days of exposure, the larvae were weighed on an analytical balance to the nearest hundredth of a mg and results recorded. The surviving larvae were then returned to new 9-cm petri dishes with 3 gm fresh untreated flour to observe subsequent pupation rates and resulting mortality rates (Ishaaya and Casida, 1975).

B. Effect of Exposure Time and Food Deprivation

This experiment was directed at determining the effect of various combinations of conditions which could occur during the practical use of flour. These include discontinuous exposure, availability or lack of food, and simulation of the possible elimination of benzoyl derivative residues from the bodies of the insects after exposure. *T. confusum* were reared as previously described in the preliminary studies. Adult beetles, about 8 days of age, were separated from the medium and transferred to petri dishes (5 x 1 cm).

For exposure tests, beetles in 3 replicates for each set of conditions were placed into dishes containing 50 mg of either 10 ppm benzoyl peroxide or diflubenzuron treated flour. This quantity of flour per dish of 5 cm diameter was sufficient to keep the beetles covered throughout the total exposure period. Beetles were unable to escape from the flour by climbing or flying.

The beetles were retained in the flour for periods of 0, 1, 2, 5, and 8 h and handled in different ways as shown in Table 1, (a, cleaned by sifting through the medium for one minute in an attempt to remove any residual derivative adhering to their bodies; b, not cleaned and returned directly to observation dishes; c, access to food immediately after exposure and handling; and d, deprived of food). Throughout the above mentioned arrangement, 108 dishes with 5 beetles per dish were set up, representing a total of 540 insects in the test.

Observations, counting, and removal of dead T. confusum were made at 24 h intervals, which represents the time elapsing between the end of a particular treatment (a - d) and the time of observation. Since these beetles feign death, it was necessary to wait for a period of time before an accurate decision could be made. Insects were considered dead when legs or other appendages did not respond to touch; those showing the slightest response were considered living. For all observations, a dissecting microscope with magnification of 4 - 60X was used (Vrba et. al., 1981).

Table 1

Effect of Exposure Time and Food Deprivation

	Treatments			
Time (h)	a	b	c	d
0	+	-	-	+
0	-	+	-	+
1	+	-	+	-
1	+	-	-	+
1	-	+	+	-
1	-	+	-	+
2	+	-	+	-
2	+	-	-	+
2	-	+	+	-
2	-	+	-	+
5	+	-	+	-
5	+	-	-	+
5	-	+	+	-
5	-	+	-	+
8	+	-	+	-
8	+	-	-	+
8	-	+	+	-
8	-	+	-	+

a, cleaned
b, not cleaned
c, access to food
d, deprived of food
+ and -, presence or absence of treatment

IV. Biochemistry

A. Chitin Dry Weight

Larvae were allowed to mature to the 5th instar stage following the treatment and exposure conditions outlined in III. A. Twenty larvae were separated from each of the 5 series of concentrations for each derivative. These larvae were homogenized and protein precipitated as outlined in I. B. For measurements of chitin in the resulting samples; the chitin was separated from the other material by digestion with 5 ml NaCl solution fro ½ h. After 6 washings in 10 ml 50% ethanol and H_2O, the chitin was dried in a hot air oven at 110°C for 3 h. The dry weight was determined to the nearest hundredth mg utilizing an analytical balance (Candy and Kilby, 1962).

B. Glucose - Quantitative and Enzymatic (Hexokinase) Determination

The hexokinase (HK) method is recognized as highly specific for glucose measurement. The procedure provided by Sigma, (Sigma Diagnostics, St. Louis, Missouri, 1984), involves the following reactions:

$$\text{Glucose} + \text{ATP} \xrightarrow[\text{Mg++}]{\text{HK}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH}$$

$$\text{NADPH} + \text{PMS} \longrightarrow \text{NADP} + \text{PMSH}$$

$$\text{PMSH} + \text{INT} \longrightarrow \text{PMS} + \text{INTH}$$

The analysis is based on the hexokinase catalyzed conversion of glucose to glucose-6-phosphate (G-6-P). This reaction is coupled with the subsequent reduction of nicotinamide adenine dinucleotide (NADP) to NADPH by action of glucose-6-phosphate dehydrogenase (G-6-PDH). In the presence of NADPH, phenazine methosulfate (PMS) is reduced. The

PMSH generated is then responsible for reduction of iodonitrotetrazolium chloride (INT) forming INTH, which is measured colorimetrically at 520 nm. The colorimetric response is proportional to the glucose concentration.

Larvae were allowed to mature to the 5th instar stage following the treatment and exposure conditions outlined in III. A. Twenty larvae were separated from the 10 ppm concentration of each derivative. These larvae were homogenized and protein precipitated as outlined in I. B. The described procedure does not differentiate between glucose and glucose-6-phosphate or other hexoses, such as fructose and mannose. The hexokinase assay has been found free of interference from a large number of contaminants. Certain drugs and other substances are known to influence levels of glucose. For this procedure a colorimeter that transmits light at 520 nm was used to measure the absorbance. Matched cuvets, specific for the instrument, were used. Automatic pipets were used to deliver 0.02, 1.0, 10.0, and 20.0 ml. Deionized H_2O was also used.

The procedure obeys Beer's law up to a glucose concentration of at least 300 mg/dl. A calibration is not required, since a standard included with each series of assays is used for calibration purposes. See appendices for protocal and calculations.

C. Micro Protein Determination (Phenol Reagent Method)

This procedure is a modified version of the Lowry procedure. The volume of specimen is reduced. The Biuret reagent is reacted with protein solution followed by phenol reagent. After development, the color is read at a suitable wavelength between 550 and 750 nm (maximum color

observed at 700-750 nm). See appendices for reagents used.

Since this method is very sensitive, the specimen is diluted so that the final protein concentration is between 15 and 100 mg/dl (150 to 1000 micrograms per ml). Dilutions can be made with sodium chloride solution. All glassware must be free of protein. This assay is dependent on the tryptophan and tyrosine content of proteins. Therefore, the presnce of either of these as free amino acid contaminants will interfere. Glycine decreases the color with protein by up to 50%. Ammonium sulfate at a final concentration above 0.15% decreases color development. Uric acid, guanine, and xanthine react with the phenol reagent, whereas quanosine does not react appreciably. Most phenols, except nitrophenol, reduces the reagent. The procedure also is affected by potassium ions, magnesium ions, EDTA, thiol reagents, tris, and some carbohydrates. A colorimeter transmitting light between 550-750 nm was used for measuring absorbance. See appendices for the establishment of a calibration curve.

There were two types of samples used in this study; 1) soluble samples - Fifteen 5th instar larvae (following exposure as outlined in III. A.) were homogenized in dH_2O using a chilled glass teflon tissue grinder. The homogenate was centrifuged for 15 minutes at 12,000 g at 2°C. The resulting pellet was redissolved in sodium chloride solution. 2) precipitated samples - The above soluble sample procedure was followed, and the sample was diluted with cold TCA (trichloroacetic acid) to a final concentration of 5-6%. The sample was centrifuged for 15 minutes at 12,000 g at 2°C to pellet the precipitated proteins. Supernatant, which contained interfering compounds, was removed and the

pellet was redissolved with dilute phosphate, 0.05M, pH 7.0. For a stepwise protocal, see appendix.

V. Histology

Larvae were allowed to mature to the 5th instar stage following the treatment and exposure conditions outlined in III. A. Several larvae were then sacrificed for histological examination in order to identify any abnormalities within the cuticle which might have resulted from treatment with the benzoyl derivatives. Larvae were processed for histological sectioning and placed in paraffin blocks for cutting. Sections were cut at 3-5 microns and placed on slides for staining with Mallory's triple connective tissue stain. See appendix for staining procedures.

RESULTS

I. Preliminary Studies

A. Thirty Day Life-cycle (Temperature and Relative Humidity)

By maintaining a temperature of 27±2°C and a relative humidity of 60±5%, the life-cycle of T. confusum was delimited to approximately 30 days. Fluctuations did occur in both temperature and relative humidity (Graph 1). These fluctuations were assumed to be the result of several conditions: inclement weather, seasonal change, or changes in internal conditions due to switch overs from heating to air-conditioning in the area where the beetles were housed. By altering either temperature or relative humidity, the length of the T. confusum life-cycle can be changed dramatically.

Table 2 presents the resulting life-cycle stages which were determined by the conditions set above. The length of stages (in days) shows an average which represents a group of thirty organisms: eggs, larvae, or pupae. Maximum and minimum figures are also seen here. The egg stage, 6th instar stage, and the pupae stage appear to be longer in length, while the first five larval instars seem to advance very rapidly. Body length, head capsule width, and weight are the three parameters which separate each advancing instar from another. In all instars, there was an increase in the parameter measured, as the beetle matured to a pupae. These results correlated very well with Brindley's (1930) findings.

Graph 1: Temperature °C and Relative Humidity

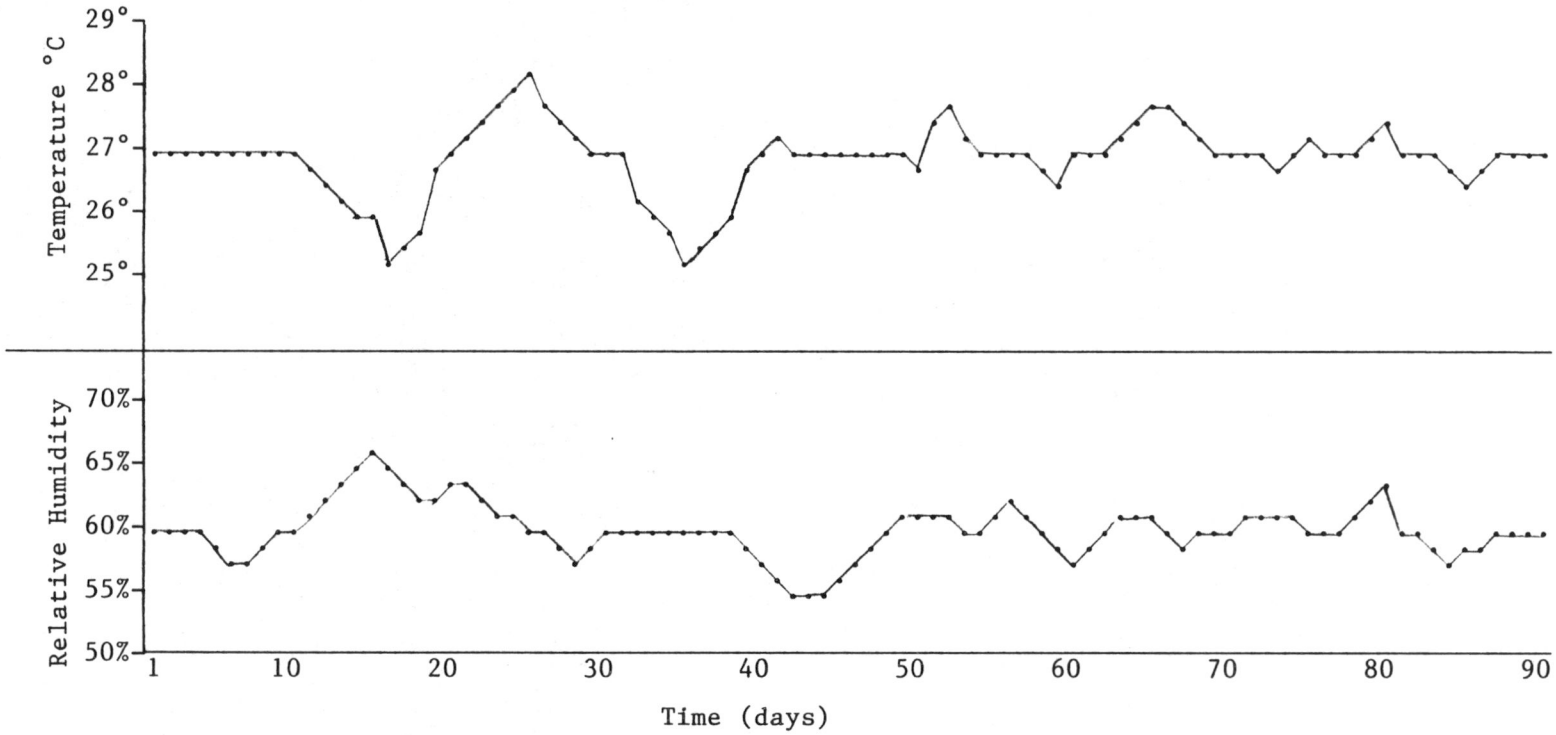


Table 2: Normal Weights and Measurements for T. confusum

Stage	Length of Stages (in days)				Body Length of Larvae (mm.)		Head Capsule Width (mm.)		Wt. (mg.)
	Min.	Max.	Ave.	Stand. Dev.	Ave.	Stand. Dev.	Ave.	Stand. Dev.	
Egg	5.5	5.5	5.5	0.00	----	----	----	----	-----
Instar 1	2.0	2.0	2.0	0.00	1.18	0.05	0.18	0.01	0.028
Instar 2	2.0	3.0	3.0	0.05	1.64	0.11	0.22	0.03	0.035
Instar 3	2.0	3.0	2.5	0.68	2.38	0.08	0.29	0.01	0.119
Instar 4	2.0	3.0	2.7	0.55	3.23	0.20	0.40	0.01	0.332
Instar 5	2.0	3.0	2.8	0.36	4.00	0.44	0.53	0.04	1.090
Instar 6	5.0	7.0	5.5	0.71	6.00	0.70	0.69	0.03	2.400
Pupa	6.0	7.0	6.2	0.44	----	----	----	----	-----
Totals	26.5	33.5	30.2						

B. Determination of the Larval Stage Most Susceptible to Treatment

Electrophoresis using polyacrylamide gels in the presence of an anionic detergent, sodium dodecly sulfate (SDS), has proven to be a useful tool for the separation of protein subunits and the determination of their molecular weights. The molecular weight of a given protein can be determined by comparing its electrophoretic mobility with known protein markers. By utilizing a protein densitometer, the percent of total protein can be measured in each protein band. Percent total protein is determined in a quantitative manner utilizing a measured peak area to give the readings in each band. Table 3 represents densitometer readings in percent total protein.

Table 3: Densitometer Readings in % Total Protein From Developmental Stages of T. confusum

	Molecular Weight Marker	Eggs	1st Instar	2nd Instar	3rd Instar	4th Instar	5th Instar	6th Instar	Pupae	Adult
Controls	205,000	40%	51%	52%	54%	58%	60%	62%	68%	70%
	116,000	30%	29%	28%	27%	25%	22%	22%	20%	20%
	97,000	30%	20%	20%	19%	17%	18%	16%	12%	10%
	68,000	---	---	---	---	---	---	---	---	---
	38,000	---	---	---	---	---	---	---	---	---
Diflubenzuron	205,000	42%	54%	52%	50%	49%	44%	22%	20%	18%
	116,000	38%	31%	29%	30%	30%	32%	46%	51%	54%
	97,000	---	---	---	---	---	---	---	---	---
	68,000	20%	15%	19%	20%	21%	20%	20%	18%	16%
	38,000	---	---	---	---	---	4%	12%	11%	12%
Benzoyl Peroxide	205,000	66%	72%	70%	60%	47%	42%	33%	30%	28%
	116,000	23%	21%	22%	32%	43%	47%	52%	55%	56%
	97,000	---	---	---	---	---	---	---	---	---
	68,000	11%	7%	8%	8%	10%	6%	10%	10%	12%
	38,000	---	---	---	---	---	5%	5%	5%	4%

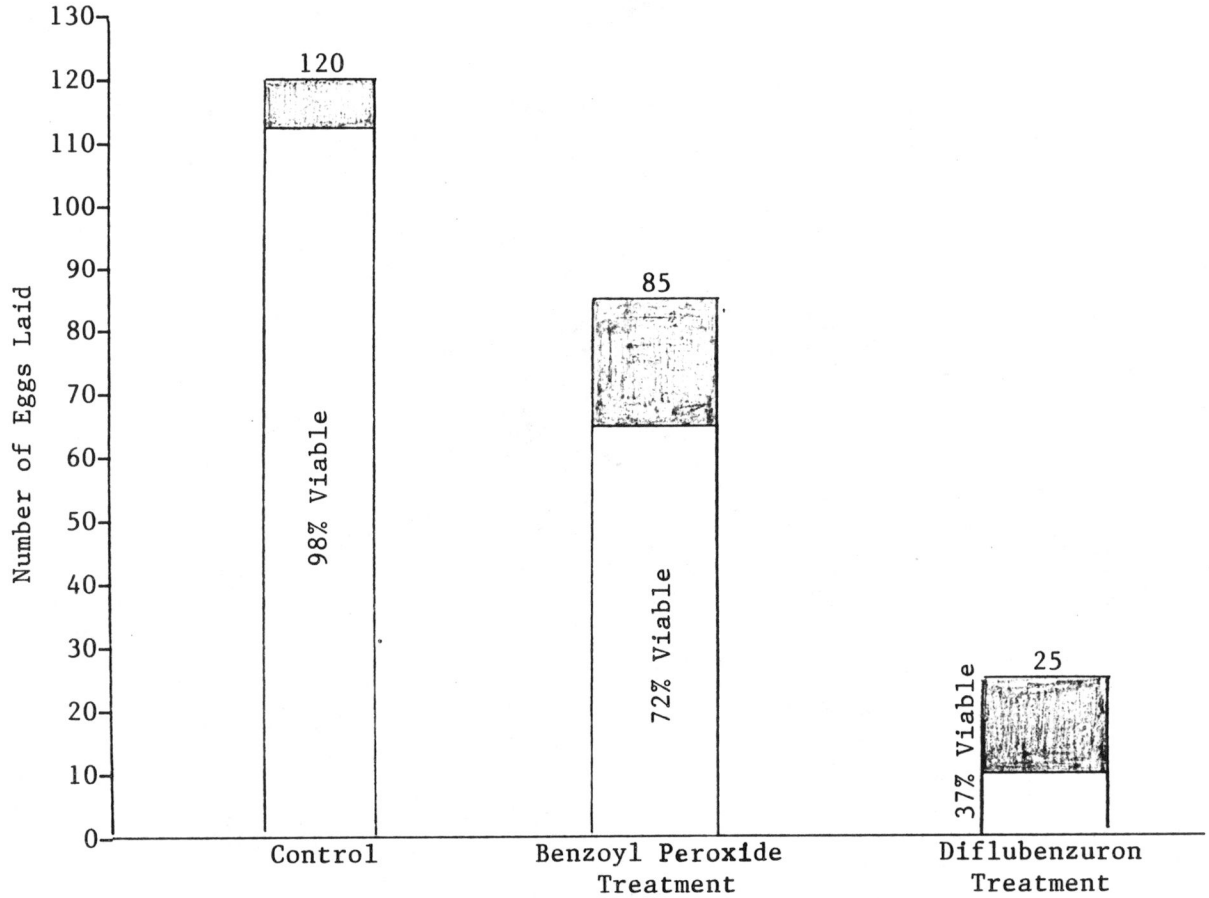
In the controls, molecular weights ranged from 205-97 Kd in all stages of development. As the beetles matured to the adult stage, the higher molecular weight proteins increased in total percentage while the lower molecular weight proteins decreased in total percentage. With both benzoyl derivatives, proteins with molecular weights of 68 and 38 Kd appeared, while those with a molecular weight of 97 Kd disappeared. It may be important to note that hexokinase, which is an essential enzyme in the pathway of glucose conversion to chitin, has a molecular weight of 96 Kd. It is also interesting that the 38 Kd protein does not appear until the 5th instar stage with either derivative. The 205 Kd protein with both derivatives shows an initial increase in percent total protein but then dramatically drops following the 2nd instar stage. The 116 Kd protein decreases slightly at first but then continues to increase following the 3rd instar stage. The 97 Kd protein is absent in all developmental stages. The 68 Kd protein seems to maintain a fairly constant protein percentage with little fluctuation as the beetle matures. The 38 Kd protein does not appear until the 5th instar stage and shows little change with benzoyl peroxide, but triples in total percentage protein with diflubenzuron. All results with the treated samples differ from those with the controls. Results with benzoyl peroxide are similar to those with diflubenzuron, but elicit lower percentages. Based on these findings, it was determined that the 5th instar stage seemed to be most susceptible to treatment and was therefore utilized as the stage to be tested in subsequent experiments.

II. Fecundity

A. Number of Eggs Laid Following Exposure

Graph 2 presents the number of eggs laid by 30 female adult beetles exposed for a 30 day period and then removed from treatment. The eggs resulted from the 3 day period following exposure. The controls, which contained 10 females, showed a total of 120 eggs, which is an average of 12 eggs per female in a 3 day period or 4 eggs per female per day. This result correlates with the egg numbers per day determined by Park (1932). Of the 120 eggs laid, 98% were viable. Following benzoyl peroxide treatment, 10 female laid 85 eggs in a 3 day period which is converted to an average of 8.5 eggs per female or 2.8 eggs per female per day. Only 72% of these eggs were viable. Following diflubenzuron treatment, 10 females laid only 25 eggs in a 3 day period. This is converted to an average of 2.5 eggs per female or 0.8 eggs per female per day. Only 37% of these eggs were viable. In comparing treatment results to the controls, there is a decrease in the total number of eggs and a concomittant decrease in egg viability going from controls to benzoyl peroxide to diflubenzuron, respectively. Benzoyl peroxide shows a 29% decrease in egg number and a 26% decrease in viability, while diflubenzuron shows a 79% decrease in egg number and a 61% decrease in viability. Benzoyl derivatives markedly affect fecundity in adult female beetles and simultaneously affect the viability of the fertilized eggs deposited in the medium.

Graph 2: Number of Eggs Laid Following Exposure



Ten female adult beetles exposed for a 30 day period and then removed from treatment. Eggs resulting over the next 3 day period following exposure.

B. Number of Viable Eggs Following Dipping

The T. confusum egg is quite sticky and adheres tenaciously to the medium it is deposited in. It has a transparent appearance when viewed under the microscope and is approxiamately 0.3 x 0.8 mm in size. Most T. confusum eggs are viable when left untreated, but following treatment for only one second in aqueous dilutions of both benzoyl peroxide and diflubenzuron, their viability changes dramatically as seen in Table 4. At 100 ppm with both derivatives, there is a resulting 100% mortality rate of dipped eggs. At 10 ppm, diflubenzuron still resulted in 100% mortality which is a four fold increase over that seen with benzoyl peroxide. At 1 ppm, benzoyl peroxide resulted in only 1.2% mortality while diflubenzuron shows an approximate 75 fold increase. At 0.1 ppm, all eggs dipped in benzoyl peroxide remained viable while diflubenzuron showed a 1.9% mortality. This was almost a 200 fold difference between treatments. At 0.01 ppm, neither derivatives showed any adverse effects on egg viability folowing dipping. The critical concentration of benzoyl peroxide most affecting egg viability was found between 10 and 100 ppm while that of diflubenzuron was between 0.1 and 1 ppm, an appropriate 100 fold difference.

Table 4: Mortality of 0-1 Day-Old Eggs Dipped In Aqueous Dilutions of Benzoyl Peroxide and Diflubenzuron

Concentrations (ppm)	Benzoyl Peroxide (% Mortality)	Diflubenzuron (% Mortality)
100	100	100
10	26.5	100
1	1.2	74.5
0.1	--	1.9
0.01	--	--

Three replications per concentration and 10 eggs per replication.

III. Environmental Parameters on Survival

A. Changes in Larval Weight, Pupation Rates, and Mortality Rates

Following 12 days of exposure, 5th instar larvae showed a more significant weight change after diflubenzuron treatment than that seen with benzoyl peroxide (Table 5). After benzoyl peroxide treatment, larvae showed a more gradual decrease in weight loss progressing from 0.01 ppm to 100 ppm. Only at the 10 and 100 ppm levels is a significant difference seen between sample means and the controls. After diflubenzuron treatment, weight loss is more severe resulting in a significant difference between sample means and the controls in every category. It is important to note that at 100 ppm, there was a resulting total mortality of all larvae and no measurements could be obtained with both derivatives. A more acute weight loss is seen at higher concentrations than at lower concentrations.

Table 5: Effect of 12 Day Exposure to Dietary Diflubenzuron and Benzoyl Peroxide on Larval Weight

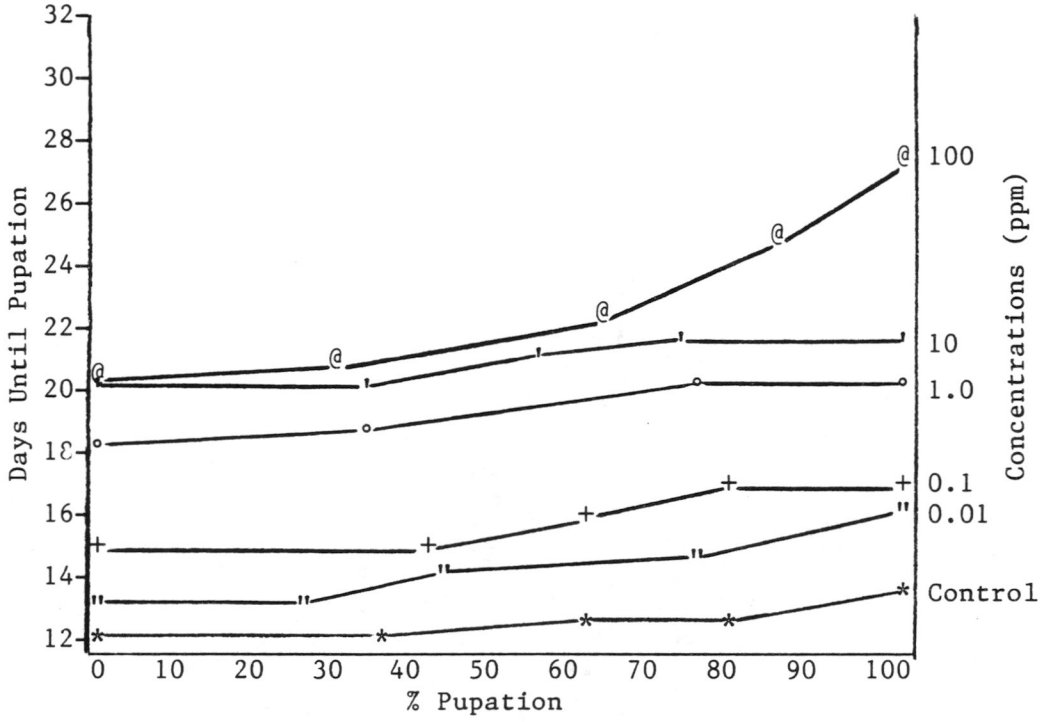
Concentrations (ppm)		0.01	0.1	1.0	10	100
Diflubenzuron	$\bar{x}$	1.20	1.15	0.98	0.82	**
	s^2	0.25	0.30	0.20	0.05	**
	t	4.06*	4.52*	7.56*	12.5*	**
Benzoyl Peroxide	$\bar{x}$	1.48	1.46	1.41	1.26	1.03
	s^2	0.13	0.21	0.08	0.16	0.14
	t	0.16	0.29	1.39	3.59*	7.38*
Controls	$\bar{x}$ = 1.49					
	s^2 = 0.21					

$\bar{x}$ = average weight in mg.
N = 90 larvae per concentration, therefore 540 larvae for total experiment
s^2 = variance
t = significant difference value between sample means
* = For the t value a 0.05 level of significance was used. Therefore when t was less than -1.960 or greater than 1.960, the average weights were significantly different from the controls.
** = no measurements due to total mortality of larvae.

The surviving larvae were then observed until pupation occurred. The resulting pupation rates can be seen in Graphs 3 and 4, and the accompanying mortality rates are in Table 6. The control group larvae began pupating at day 12 and continued until pupation was complete around day 14. With the increasing benzoyl derivative concentrations there is a delay in the onset of pupation in a linear fashion. There is also an increase in the period of time it requires for pupation to reach completion. The higher the concentration, the longer it takes for the onset of pupation to occur. The disparity between test compounds became more prominent at higher concentrations. A similar relationship can be seen in the mortality rates (Graph 5). As concen-

trations increased, both derivatives evoked a higher mortality rate; diflubenzuron results are more significant than benzoyl peroxide and result in total mortality at 100 ppm.

Graph 3: Pupation Rates Following Treatment With Benzoyl Peroxide



Graph 4: Pupation Rates Following Treatment With Diflubenzuron

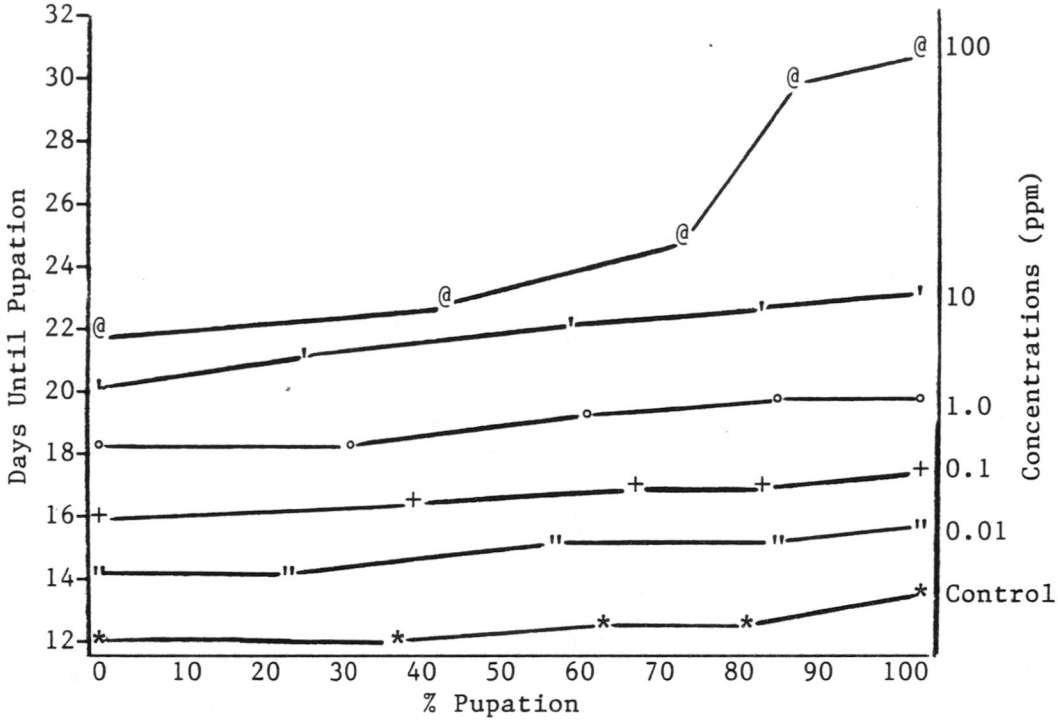


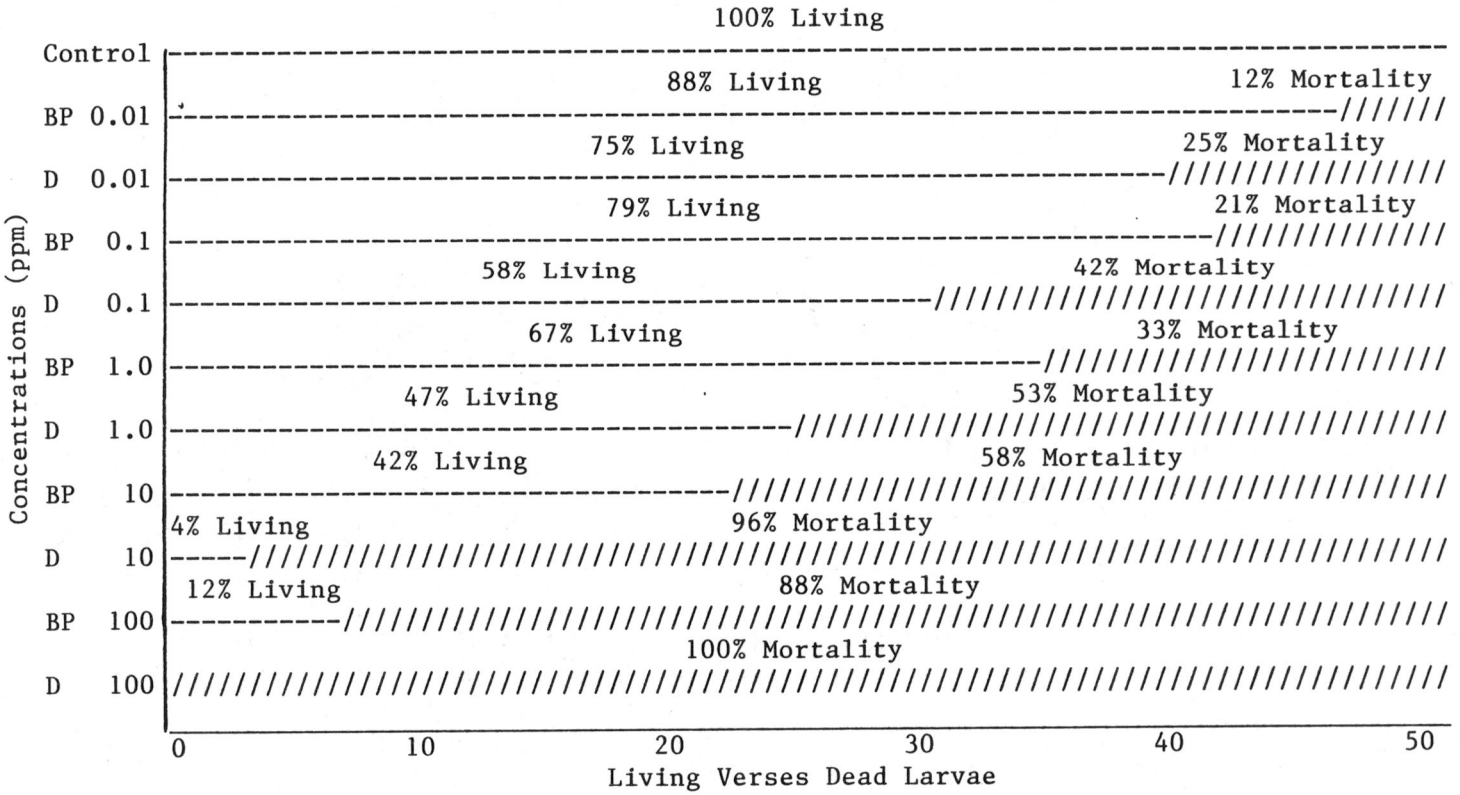
Table 6: Pupation Rates of Viable Larvae Following Treatment

Concentrations (ppm)	0.01	0.1	1.0	10	100
Benzoyl Peroxide	79(12)	71(21)	60(33)	38(58)	11(88)
Diflubenzuron	68(25)	52(42)	42(52)	4(96)	2(98)

N = 90 larvae per concentration
The number outside the parenthesis is the number of larvae that pupated out of 90.
The number in the parenthesis represents the mortality rate at that concentration.

Graph 5

Mortality Rates of 5th Instar Larvae Following Treatment With Diflubenzuron and Benzoyl Peroxide



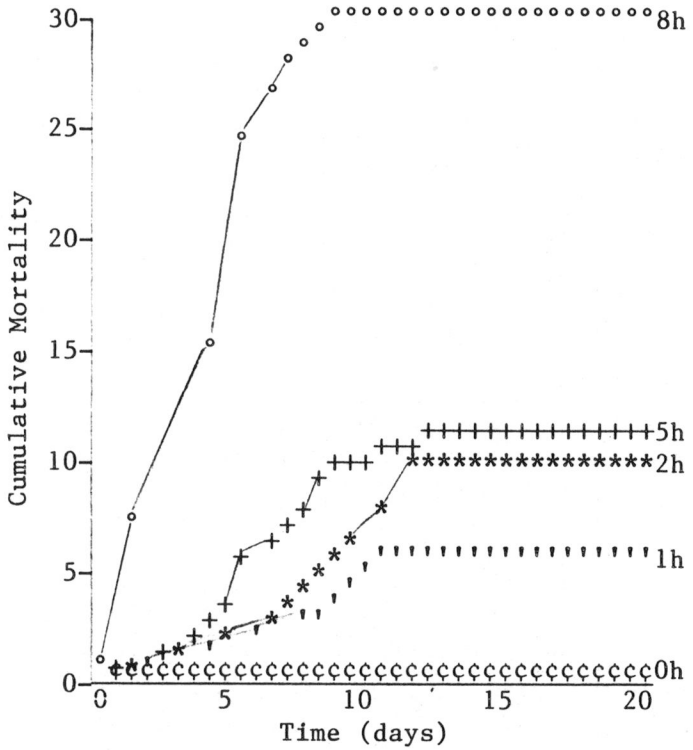
BP = Benzoyl Peroxide, D = Diflubenzuron, -- = number living, // = number dead

B. Effect of Exposure Time and Food Deprivation

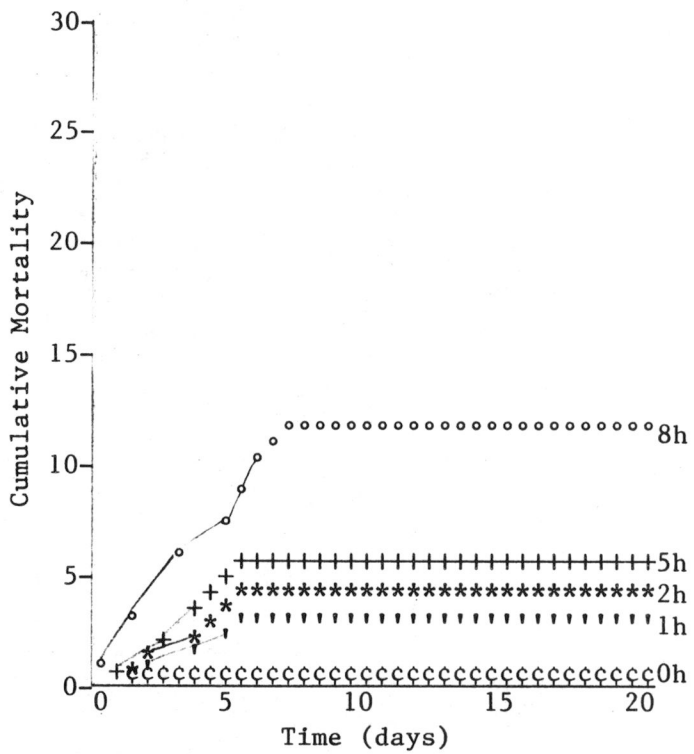
Results are presented in the form of graphical interpretation. In summary, Graphs 6-13 generally reveal that cumulative mortality increased with increased exposure time. Insects exposed to the derivative without access to food after exposure showed continued mortality (Graphs 6-9), but mortality leveled off at much lower levels than when insects were allowed to feed (Graphs 10-13). The actual sifting of insects to remove any residues adhering to their bodies, did not seem to alter the mortality rates with either treatment. Access to food following exposure, caused an increase in the resulting mortality rates, comparing Graphs 6-9 with Graphs 10-13. Diflubenzuron treatment evoked a much higher cumulative mortality when the beetles were allowed to feed. Therefore, it was concluded that the act of feeding allowed the derivatives to be ingested and subsequently lead to death, whereas any residues on the body surfaces did not appear to affect the beetles existence. It is interesting to note that in Graphs 10 and 12 at all exposure times, diflubenzuron treatment led to total mortality following feeding.

Empirical Cumulative Mortality Rates and Theoretical Mortality Curves of T. confusum After 0, 1, 2, 5, and 8 H of Exposure, Not Sifted and Deprived.

Graph 6: Diflubenzuron

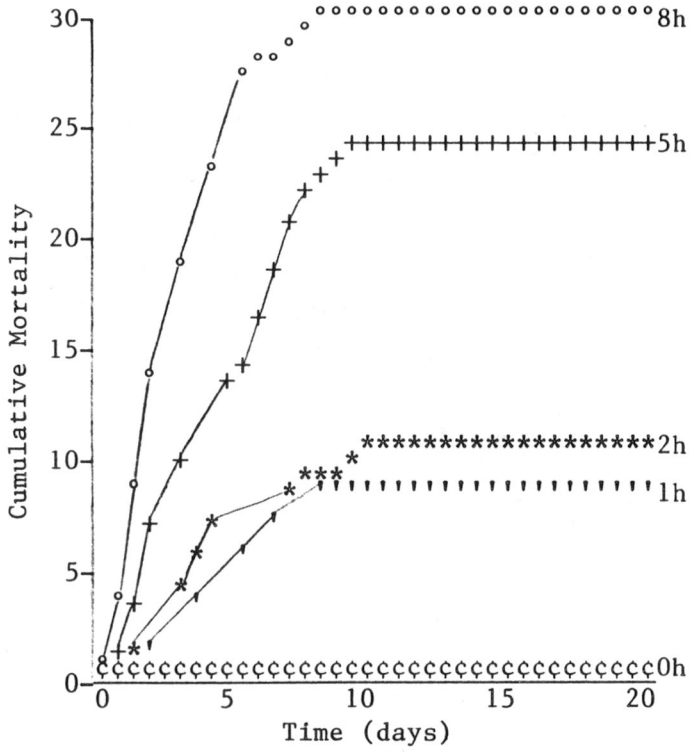


Graph 7: Benzoyl Peroxide

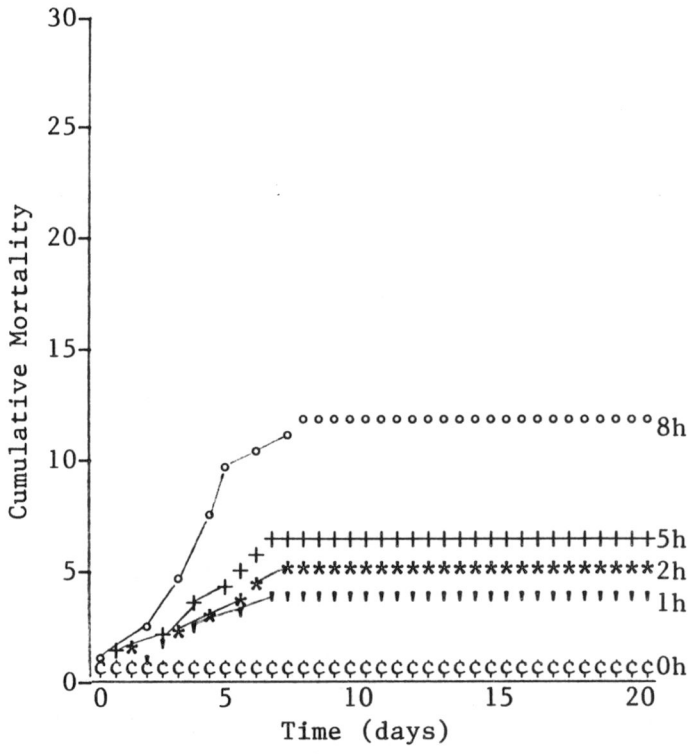


Empirical Cumulative Mortality Rates and Theoretical Mortality Curves of T. confusum After 0, 1, 2, 5, and 8 H of Exposure, Sifted and Deprived.

Graph 8: Diflubenzuron

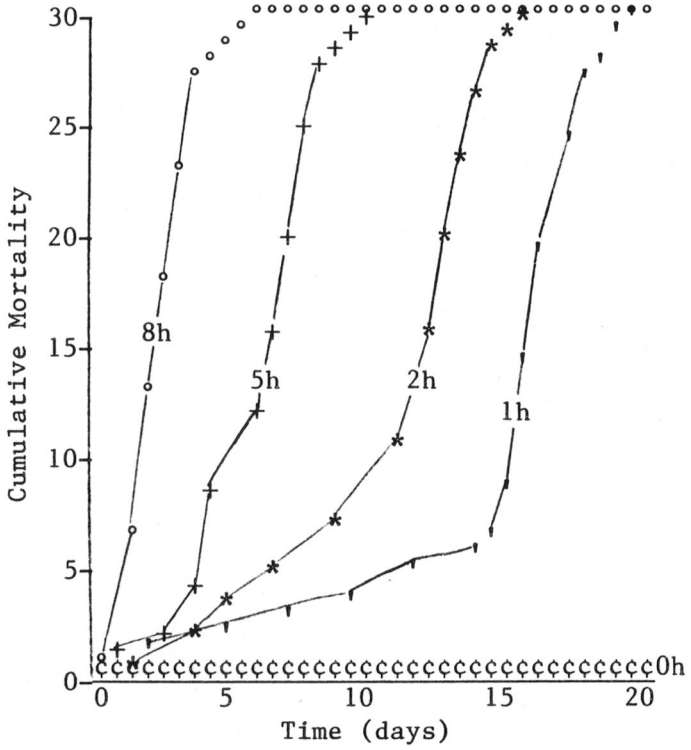


Graph 9: Benzoyl Peroxide

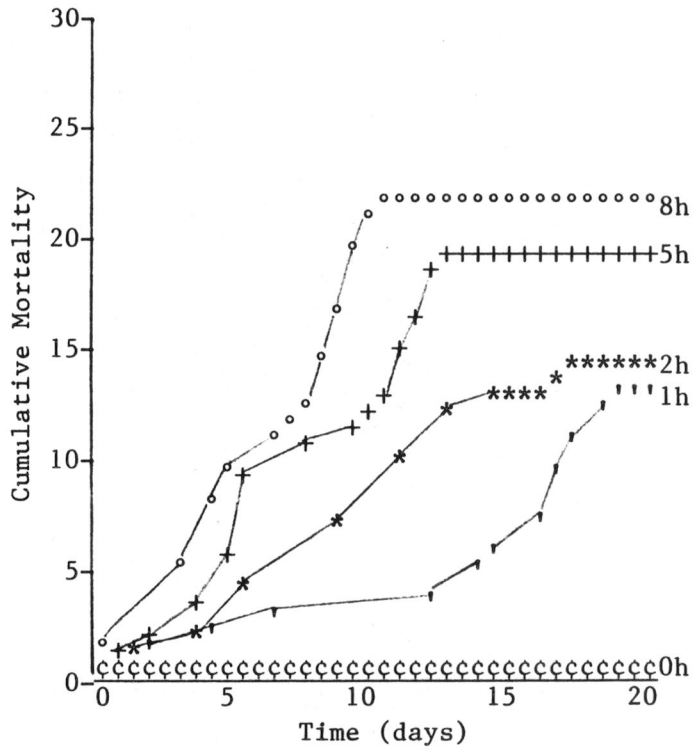


Empirical Cumulative Mortality Rates and Theoretical Mortality Curves of T. confusum
After 0, 1, 2, 5, and 8 H of Exposure, Not Sifted and Fed.

Graph 10: Diflubenzuron

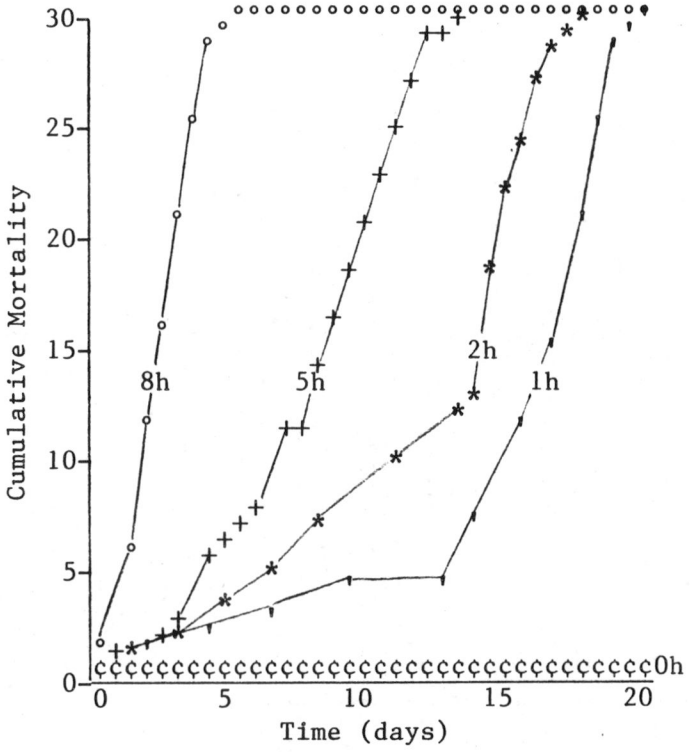


Graph 11: Benzoyl Peroxide

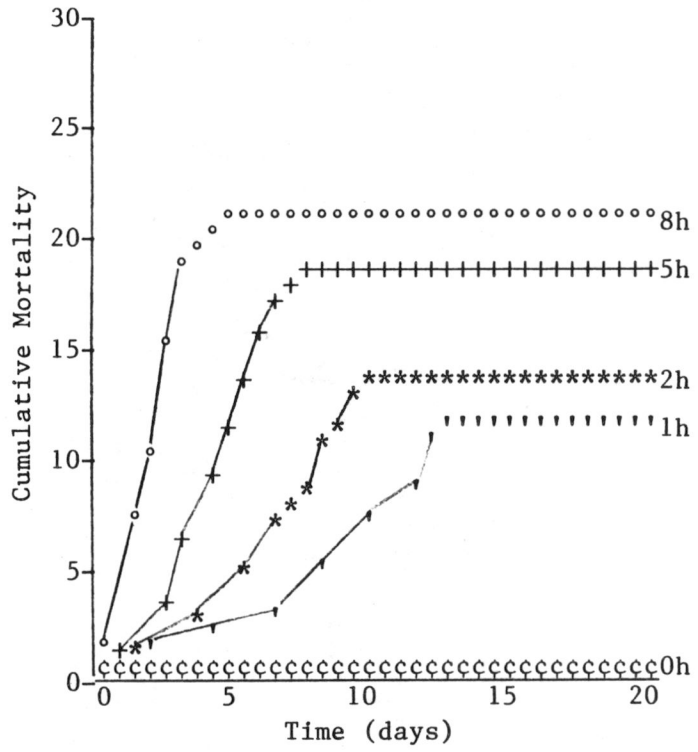


Empirical Cumulative Mortality Rates and Theoretical Mortality Curves of T. confusum
After 0, 1, 2, 5, and 8 H of Exposure, Sifted and Fed.

Graph 12: Diflubenzuron



Graph 13: Benzoyl Peroxide



IV. Biochemistry

A. Chitin Dry Weight

Chitin formation in T. confusum begins at the 1st instar stage and continues through the first few days of adult life, but as determined by Candy and Kilby (1962), the largest amount of chitin is deposited at the 5th instar stage. Table 7 shows the variation in the total chitin content of a group of 5th instar larvae following exposure to both diflubenzuron and benzoyl peroxide. Once again benzoyl peroxide treatment promoted a gradual decrease in chitin content as concentrations increased; significant differences are only seen at 10 and 100 ppm. Following diflubenzuron treatment, chitin content dramatically decreased until total mortality resulted at 100 ppm. All diflubenzuron concentrations resulted in significantly different chitin amounts as compared to the controls. Based on this data it seemed apparent that benzoyl derivative exposure may somehow interfere with chitin deposition in T. confusum larvae, which became more and more critical as these beetles reached adulthood.

Table 7: Exposure of Dietary Diflubenzuron and Benzoyl Peroxide on Chitin Dry Weight of 5th Instar Larvae

Concentrations (ppm)		0.01	0.1	1.0	10	100
Diflubenzuron	$\bar{x}$	0.73	0.61	0.42	0.08	**
	s^2	0.05	0.12	0.23	0.17	**
	t	3.43*	4.22*	5.24*	9.00*	**
Benzoyl Peroxide	$\bar{x}$	0.98	0.93	0.87	0.49	0.39
	s^2	0.20	0.16	0.08	0.14	0.21
	t	0.28	0.79	1.60	5.29*	5.66*
Controls	$\bar{x}$ = 1.01 s^2 = 0.15					

N = 30 larvae per concentration

B. Glucose - Quantitative and Enzymatic (Hexokinase) Determination

Without a sufficient quantity of glucose present in the developing T. confusum larvae, chitin formation becomes hindered (see Figure 4). Table 8 presents the quantitative amounts of glucose existing in 5th instar larvae following exposure to 10 ppm diflubenzuron and benzoyl peroxide. Both derivatives resulted in a decrease in glucose content but diflubenzuron seemed more pronounced. Benzoyl peroxide resulted in a 26% decrease and diflubenzuron resulted in a 42% decrease. Therefore, since less glucose is present, less chitin can be formed, which in turn can lead to death or other developmental abnormalities.

Table 8: Glucose - Quantitative Analysis

	Control	Benzoyl Peroxide	Diflubenzuron
Glucose (mg/dl)	127.0	94.0	74.0
Glucose (mmol/l)	7.06	5.21	4.12
% Decrease	-----	26%	42%

For calculations, see appendix.

C. Micro Protein Determination (Phenol Reagent Method)

As seen in Graph 14 and Table 9, the overall protein content is also affected by treatment with benzoyl peroxide and diflubenzuron. These results indicated that there is a decrease in the total protein content of the 5th instar larvae as a result of treatment. Diflubenzuron had a more substantial affect by decreasing the total protein concentration by more than 50% over that seen in the controls in both the soluble and precipitated samples;

Soluble sample - benzoyl peroxide 20% decrease

diflubenzuron 60% decrease

Precipitated sample - benzoyl peroxide 26% decrease

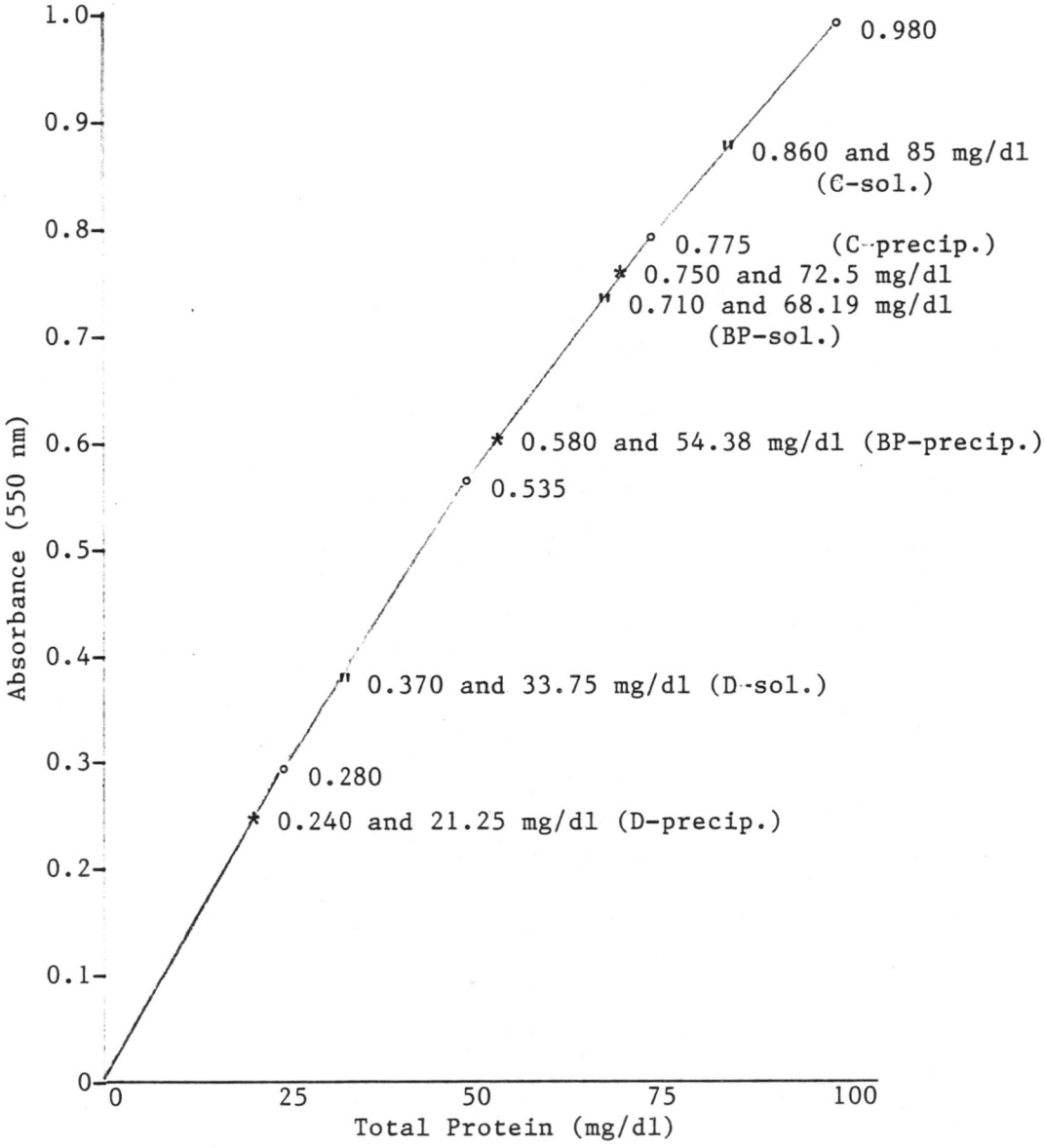
diflubenzuron 71% decrease

Benzoyl peroxide also had an effect on the total protein concentration but to a lesser extent than that seen with diflubenzuron. The effect of precipitating the samples before the protein assay showed an approximate decrease in the total protein concentration of 15%. Protein is lost by this margin due to the protein precipitation procedure, but there is a larger decrease in the total protein concentration by both of the benzoyl derivatives.

Table 9: Micro Protein Analysis

Samples	Protein Concentration	% Decrease
Soluble:		
Control	85 mg/dl	---
Benzoyl Peroxide	68.13 mg/dl	20%
Diflubenzuron	33.75 mg/dl	60%
Precipitated:		
Control	72.5 mg/dl	---
Benzoyl Peroxide	54.38 mg/dl	26%
Diflubenzuron	21.25 mg/dl	71%

Graph 14: Micro Protein Analysis



There was a 10X dilution factor with each sample.

V. Histology

Histological examination, as seen in Figures 6-8, revealed an abnormal development of the 5th instar larvae following treatment with 10 ppm diflubenzuron and benzoyl peroxide. Figure 6 shows a cross-section at 10X magnification through the mid portion of a normal 5th instar larvae. Figure 7 shows a similar cross-section through a 5th instar larvae treated with 10 ppm benzoyl peroxide. Figure 8 shows the same cross-section through a 5th instar larvae treated with 10 ppm diflubenzuron. Note the significant changes which occur not only in the chitinous endocuticle but also in the internal structures.

In both treatments the exoskeleton appeared weakened and more porous than that seen in the normal cross-section. The endocuticular thickness remains constant in the normal cross-section but this thickness is greatly diminished in both treated sections. Blocking of the endocuticular growth probably resulted in an integument which is too weak to maintain the normal appearance with increasing internal pressure during larval growth. This blocking action was probably caused by interference with chitin synthesis in preceding larval stages. Distortions that are visible in the treated sections are apparently coagulated material which has been transformed by the benzoyl derivative treatment. The very fragile structures seen in the diflubenzuron section probably are the transgressors leading to eventual mortality for these treated larvae. See appendix for histological protocals and staining procedures.

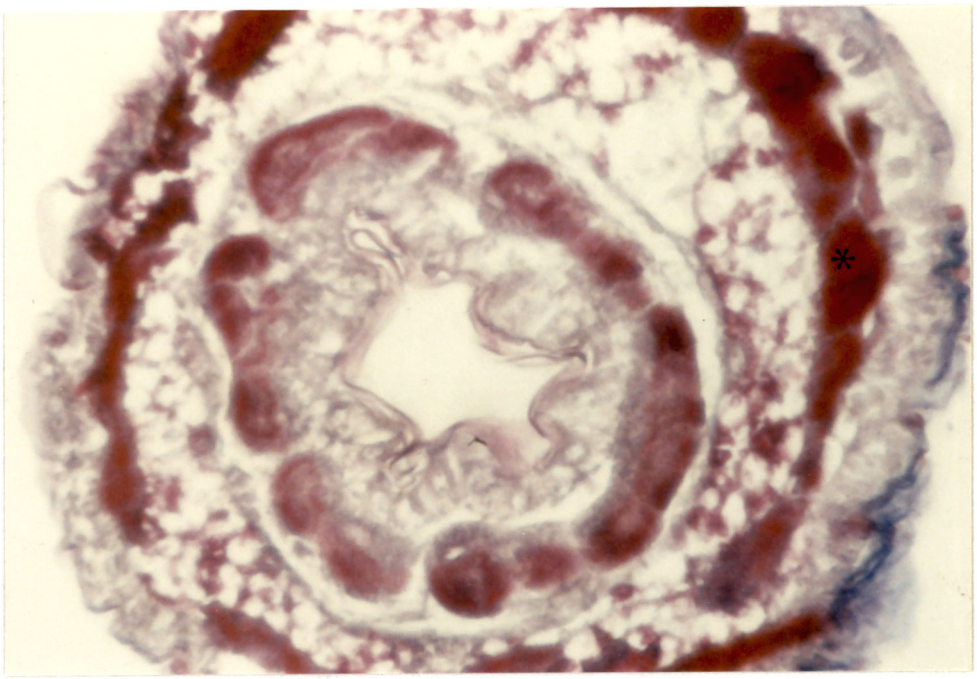


Figure 6: Untreated 5th instar larvae mid section, 10X magnification, Mallory's Triple stain
* Chitin (red)

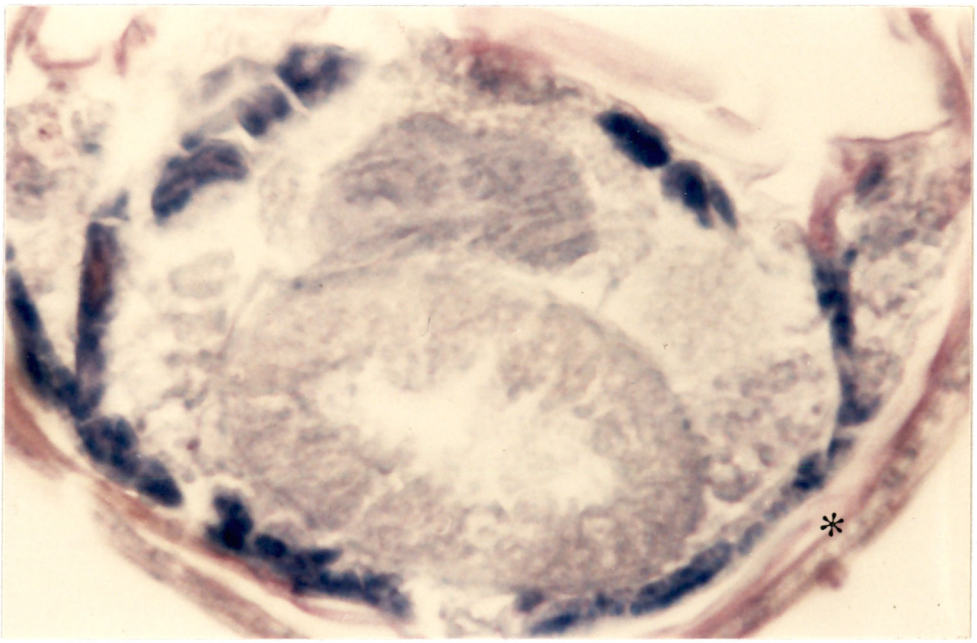


Figure 7: 5th instar larvae mid section treated with 10 ppm benzoyl peroxide, 10X magnification, Mallory's Triple stain
* Chitin (red)

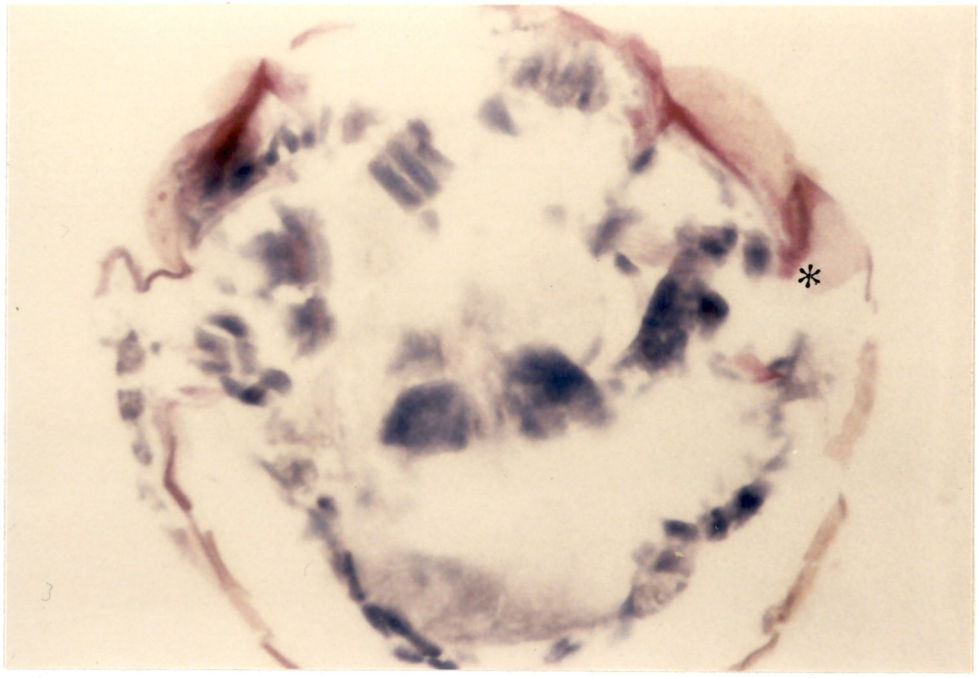


Figure 8: 5th instar larvae mid section treated with 10 ppm diflubenzuron, 10X magnification, Mallory's Triple stain
* Chitin (red)

DISCUSSION

Based on preliminary studies it may be concluded that the existence of T. confusum is very dependent on the environment in which it resides. Temperature and relative humidity fluctuations can cause a vasilation in the length of the life-cycle, which is also supported by Chapman (1928). With a constant temperature of 27±2°C and 60±5% relative humidity, it was easy to maintain a stock culture which demonstrated a 30 day life-cycle. As the beetles progressed through the larval stages, it was confirmed based on electrophoretic findings, that the 5th instar stage was the most susceptible to treatment with both benzoyl peroxide and diflubenzuron. Mulder and Gijswijt (1973) stated that the 5th instar stage in the development of T. confusum was the most metabolically active and therefore, the most susceptible to any change in its environment. From Table 3, it is quite obvious that the metabolic activity of the entire life-cycle of T. confusum is considerably affected by exposure to both benzoyl derivatives, especially at the 5th instar stage. At this stage new 38 Kd proteins begin to appear. Larger molecular weight proteins are decreasing in number while smaller weight proteins seem to be expressed. This suggests that the smaller subunits are not being assimilated into the larger building proteins, but the smaller proteins are being integrated, therefore only certain steps in the protein metabolic pathways are being altered. Hexokinase has a molecular weight of around 96 Kd and is essential to chitin biosynthesis. With

both benzoyl derivatives, there is an absence of the normally appearing proteins around the 97 Kd level. This might indicate that hexokinase is being inhibited. This metabolic alteration is further supported by the biochemical assays involving glucose and protein anabolism. Glucose quantities are reduced by 20% with benzoyl peroxide and over 40% with diflubenzuron. Protein quantities are reduced by similar amounts. Because both glucose and protein build-up are essential to chitin biosynthesis, this depletion of metabolic material indicates that chitin production must be altered. Data in Table 7 supports this assumption by demonstrating a gradually decreasing chitin dry weight content following exposure. As a consequence of reduced chitin deposition, the endocuticle and exoskeleton become weakened and fragile as seen in Figures 7 and 8. The devitalized integument is too weak to maintain a normal appearance with increasing internal pressure during larval growth. Thus, larval development is hindered, pupation rates are delayed (Graphs 3 and 4), and mortality rates increase (Graph 5) as the larvae endures the increased pressures of maturation. Weight loss occurs (Table 5) as the benzoyl derivatives transform the fundamental processes of metabolism. It seems that every aspect of the development process is affected by treatment with either derivative. Even adults, who no longer must endure the dramatic metamorphic changes of adolescence, appear to be affected. Fecundity decreases with a concomitant decrease in egg viability (Graph 2). Therefore, population numbers plummet. The eggs, which remain unattended, are vulnerable to the conditions present in the medium. Good (1933) sup-

ports this statement by his permeability findings, in which even a gaseous contact with insecticidal agents leads to detrimental effects in the egg. By exposing fertilized eggs to aqueous dilutions of either derivative for just one second, results in a pronounced increase in mortality (Table 4). Adults do not seem to be effected by benzoyl derivative residues adhering to their body surfaces as demonstrated in Graphs 6-13, which supports the findings of Park (1932), who noted that the exoskeleton acts as an impermeable barrier to environmental hazards. But once the benzoyl derivatives are ingested, their detrimental effects become unmistakable (Graphs 10-13).

In conclusion, the data presented from this study, supports the fact that the growth and development of T. confusum are sensitive to both diflubenzuron and benzoyl peroxide at levels as low as 1 ppm. The effects resulting from benzoyl peroxide are just as damaging as diflubenzuron, but are less severe in the final outcome. These derivatives appear to have a double effect on the larvae: death at the larval apolytic stage, and retardation of larval development which seems to result primarily from disturbances in chitin build-up. Previous studies have shown that diflubenzuron and benzoyl peroxide manifest deleterious effects in insects, but not to the extent found here. Based on the present findings, there is a promising speculation on the use of these benzoyl derivatives as possible insect control agents against larval stages of many species belonging to the Coleopterans. Their questionable use as grain protectants against insect infestation should be reconsidered in light of the results of this study, but

further investigation is necessary in order to define the exact metabolic mechanism being altered by their utilization.

CONCLUSIONS

1. Environmental parameters have a substantial effect on the extent of the life-cycle of T. confusum.

2. Protein changes occur in the 5th instar larvae as a result of exposure to both benzoyl peroxide and diflubenzuron, which can either lead to morality or other developmental abnormalities.

3. Following exposure to benzoyl peroxide and diflubenzuron, larger proteins do not seem to metabolize, resulting in the accumulation of smaller protein subunits.

4. The 5th larval instar seems to be the most susceptible to treatment with benzoyl derivatives.

5. Exposure to benzoyl derivatives is detrimental to adult fecundity, resulting in fewer eggs and a concomittant reduction in egg viability.

6. Direct surface contact with either derivative to 0-1 day-old eggs can lead to noneclosion of larvae, especially between the concentrations of 1-100 ppm.

7. More acute weight loss in 5th instar larvae occurs at higher concentrations of either derivative than at lower concentrations.

8. Following exposure there is a delay in the onset of pupation as well as a prolonged period of time for the completion of pupation once it is initiated.

9. Cumulative mortality increases with increased exposure time.

10. The mechanical sifting of insects to remove residuues from their body surfaces does not seem to affect mortality, but the digestion of the compounds by actual feeding is detrimental.

11. Chitin deposition is greatly altered, which probably results in an integument which is too weak to maintain the normal appearance of the larvae.

12. Biochemical changes in glucose and protein levels lead to modifications in metabolic pathways within the larvae which are crucial to development and maturation.

13. Histological examination reveals a weakened endocuticle which can not withstand the increasing pressures of larval growth and ultimately leads to extinction of the organism.

14. Benzoyl peroxide is very effective incontrolling the development of *T. confusum*.

15. Benzoyl peroxide's manifestations are similar but not as deleterious as those seen with diflubenzuron.

16. The potential use of both benzoyl peroxide and diflubenzuron as insect control agents is advocated based on the results of this study.

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APPENDIX A

Gel Preparation

The equipment and chemicals used in this study were: Bio-Rad Protean II Slab cell, Bio-Rad 1.5 mm spacers and 15 well combs, Ephortec 500 volt power supply, Sigma SDS molecular weight markers (kit # SDS 200), and Helena Lab Quik Quant 3 densitometer. Components for gels were prepared as follows;

1) Acrylamide/Bis (30% T, 2.67% C)
   146 gm acrylamide (29.9 gm/100 ml)
   4 gm N'N-Bis-methylene-acrylamide (0.8 gm/100 ml)

$$\%T = \frac{\text{gm Acrylamide + gm Bis-acrylamide}}{\text{Total Volume}} \times 100$$

$$\%C = \frac{\text{gm Bis-acrylamide}}{\text{gm Acrylamide + gm Bis-acrylamide}} \times 100$$

   Make to 500 ml with dH_2O. Filter and store at 4°C in the dark (30 days maximum).

2) 1.5M Tris-HCl, pH 8.8
   54.45 gm Tris base (18.15 gm/100 ml)
   150 ml dH_2O
   Adjust to pH 8.8 with 1 N HCl. Make to 300 ml with dH_2O and store at 4°C.

3) 0.5M Tris-HCl, pH 6.8
   6 gm Tris base
   60 ml dH_2O
   Adjust to pH 6.8 with 1 N MCl. Make to 100 ml with dH_2O and store at 4°C.

4) 10% SDS
   Dissolve 10 gm SDS in H_2O with gentle stirring and bring to 100 ml with dH_2O.

5) Sample Buffer (SDS reducing buffer) (store at room temperature)

dH_2O	4.0 ml
0.5M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
2 B-mercaptoethanol	0.4 ml
0.05% Bromophenol blue	0.2 ml
	0.8 ml

6) 5X Electrode (Running) Buffer, pH 8.3 (enough for 10 runs).

Tris base	45 gm (15 gm/1)
Glycine	216 gm (72 gm/1)
SDS	15 gm (5 gm/1)
	to 3 liters with dH_2O

Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 300 ml 5X stock with 1200 ml dH_2O for one electrophoresis run.

The calculated volume (in mls) required per gel slab which was 1.5 mm thick and 16 cm long is 38.4 ml. This volume will completely fill one gel sandwich to the top of the plates. The following component volumes are necessary in order to pour a Protean II (7.5%) slab gel;

dH_2O	48.5 ml
1.5M Tris-HCl, pH 8.8	25.0 ml
10% SDS	1.0 ml
Acrylamide/Bis stock	25.0 ml
10% Ammonium persulfate	0.5 ml

TEMED	0.05 ml
Total Monomer	100.0 ml

The 7.5% gel was cast in the following manner;

1) Prepare the monomer solution. Combine all reagents except APS and TEMED. Degas under vaccuum for at least 15 minutes.

2) Place 15 well comb in the glass sandwich so that the teeth are titlted at approximately a 10° angle. Further details concerning setting up the Protean II can be found in the literature which accompanies the electrophoretic unit.

3) Add APS and TEMED to the degassed monomer solution and use a pipet and bulb to pour the solution down the spacer naerest to the upturned side of the comb. Pour until the bottoms of all teeth are covered. Then adjust the comb to its prpoer position. Add monomer solution to fill the sandwich completely. No overlay solution is necessary.

4) Let the gel polymerize for 45 minutes to 1 h. The gel is now ready to load and run. Remove the comb and rinse thoroughly with dH_2O.

APPENDIX B

Sample Preparation and Loading On Gel

The samples to be tested were prepared as follows

1) Sonicate 20 - 30 eggs, larvae, pupae, or adults mixed with 5 ml dH_2O for 15 minutes.

2) Add dry $(NH_3)_2SO_4$ with magnetic stirring until protein precipitates. This has to be added slowly so that the $(NH_3)_2SO_4$ does not collect at the bottom of the beaker.

3) Adjust to 50% saturation with NaCl.

4) Add several hundred microliters of "cold" TCA, trichloroacetic acid, for a final concentration of 5 -6% TCA.

5) Spin down solution in microtubes.

6) Remove supernatant which contains interfering compounds.

7) Redissolve pellet with dilute phosphate buffer 0.05M, pH to 7.0.

8) Dilute sample at least 1:4 with sample buffer, and heat at 95°C for 4 minutes. Sample is now ready to place on gel.

Inorder to load the sample wells the following steps must be taken;

1) Prepare 1.5 liters of electrode buffer. Set aside 350 ml for the upper buffer chamber.

2) Place the remainder of the electrode buffer into the lower buffer chamber. Lower the central cooling core into the lower buffer chamber at a slight angle to prevent air entrapment under the gel sandwich. Dilute the lower buffer with dH_2O to a level of 1 cm above the bottom of the gel plate. Be sure to mix the lower buffer well with a stir bar on a magnetic stirrer.

3) Pour 350 ml of electrode buffer into the upper buffer chamber.

4) Load approximately 220 microliters of the sample into the wells under the electrode buffer with a Hamilton syringe. Insert the syringe to about 1 - 2 mm from the well bottom before delivery. Be sure to slowly deliver the sample into the wells.

APPENDIX C

Colorimetric Methods For Glucose and Protein

For the Glucose - Quantitative and Enzymatic Determination, the reagents used are as follows:

1) Glucose color reagent - INT 3.95 mmol/l and PMS 1.63 mmol/l. Store in refrigerator (2 - 6°C).

2) Glucose enzyme reagent - Store in refrigerator (2 - 6°C).

3) Glucose assay reagent is prepared by reconstituting 1 vial of glucose enzyme reagent with 17.0 ml H_2O. After adding 4.0 ml glucose color reagent invert to mix. Do not shake. The solution contains ATP 952 micromol/l, NADP 190 micromol/l, magnesium 476 micromol/l, HK 762 U/l, G-6-PDH 381 U/l, INT 0.75 mmol/l, PMS 0.3 mmol/l, buffers and stabilizers. Reagent is stable for 6 h at room temperature (18 - 26°C), for 3 days in refrigerator (2 - 6°C) or at least a week frozen.

4) Glucose standard - glucose 100 mg/dl (5.56 mmol/l) in saturated benzoic acid solution. Store in refrigerator (2 -6°C).

5) HCl, 0.1 N - prepare by diluting 8.6 ml concentrated HCl (ACS grade) to 1000ml with H_2O in a volumetric flask.

The actual manual protocal is as follows;

1) To tube labeled blank add 0.02 ml H_2O and 1.0 ml glucose assay reagent. To tube labeled standard add 0.02 ml glucose standard and 1.0 ml glucose assay reagent. To tubes labeled tests add 0.02 ml specimen abd add 1.0 ml glucose assay reagent. Mix by gently stirring and allow mixtures to stand 5 - 10 minutes at room temperature (18 - 26°C).

2) To each tube add 10.0 ml 0.1 N HCl and mix.

3) Transfer mixtures to cuvet and read absorbances (A) at 520 nm of standard and tests vs blank as reference. It is important to complete readings within 30 minutes. When glucose value exceeds 300 mg/dl, dilute tests mixture with an equal volume of 0.1 N HCl, repeat absorbance measurement and multiply by 2. Anew blank and standard must be prepared with each series of assays. The color of the blank increases with time.

The two calculations involved are the following;

$$\text{Glucose (mg/dl)} = \frac{A_{\text{Test}}}{A_{\text{Standard}}} \times 100$$

$$\text{Glucose (mmol/l)} = \frac{A_{\text{Test}}}{A_{\text{Standard}}} \times 5.56$$

Concentrations are based on glucose standard (100 mg/dl or 5.56 mmol/l).

$A_{\text{Standard}} = 0.425$

Control $A_{\text{Test 1}} = 0.540$ Glucose (mg/dl) $= \frac{0.540}{0.425} \times 100 = 127.0$

Glucose (mmol/l) $= \frac{0.540}{0.425} \times 5.56 = 7.06$

Benzoyl Peroxide $A_{\text{Test 2}} = 0.398$

Glucose (mg/dl) $= \frac{0.398}{0.425} \times 100 = 94.0$

Glucose (mmol/l) $= \frac{0.398}{0.425} \times 5.56 = 5.21$

Diflubenzuron $A_{\text{Test 3}} = 0.315$

Glucose (mg/dl) $= \frac{0.315}{0.425} \times 100 = 74.0$

Glucose (mmol/l) $= \frac{0.315}{0.425} \times 5.56 = 4.12$

For the Micro Protein Determination (Phenol Reagent Method) the reagents used are the following:

1) Biuret reagent - cupric sulfate 0.75 mmol/l and sodium hydroxide 94 mmol/l. Also contains tartrate, iodine, and carbonate. Store in refrigerator (2 -¢°C).

2) Folin and Ciocalteu's reagent - 2.0 N. store at room temperature (18 -26°c).

3) Protein standard - albumin (bovine) 10 gm/dl in sodium chloride solution 0.85%. Sodium azide 0.5% added as preservative. Store in refrigerator (2 - 6°C).

4) Sodium chloride solution 0.85 gm/dl - dissolve 8.5 gm sodium chloride in 1 liter dH_2O. Store at room temperature (18 - 26°C).

In order to establish a calibration curve follow these steps;

1) Pipet 0.5 ml of protein standard into a 50 ml volumetric flask. Dilute to 40 ml with sodium chloride solution.

2) Pipet into 5 test tubes the solutions indicated in columns 2 and 3 below.

1 Test Tube	2 Diluted Protein Standard Step 1 (ml)	3 Sodium Chloride Solution (ml)	4 Protein (mg/dl)
1	0.00	0.20	0
2	0.05	0.15	25
3	0.10	0.10	50
4	0.15	0.05	75
5	0.20	0.00	100

3) Add to each tube 2.2 ml Biuret reagent. Mix well and allow to stand at room temperature (18 -26°C) for 10 minutes.

4) Add to each tube 0.1 ml Folin and Ciocalteu's phenol reagent. Mix each tube well immediately after addition.

5) Allow to stand at room temperature (18 - 26°C) for 30 minutes.

6) Transfer to cuvets and read absorbance at 550 - 750 nm, using tube 1 as reference.

7) Plot the absorbance values versus protein concentration (column 4). A curved line passing through the origin should be obtained. The procedure becomes insensitive above protein concentrations of 100 mg/dl. Protein concentrations above this level can be assayed by diluting sample appropriately with sodium chloride solution.

In a step-wise procedure;

1) Dilute the test sample with sodium chloride solution so that the final protein concentration is between 15 and 100 mf/dl.

2) Label two or more small test tubes; blank, test 1, test 2, etc.

3) To blank, add 0.2 ml sodium chloride solution.

4) To tests, add 0.2 ml diluted test sample solution prepared in step 1.

5) Add to each 2.2 ml Biuret reagent. Mix well and allow to stand at room temperature (18-26°C) for 10 minutes.

6) Add 0.1 ml Folin and Ciocalteu's reagent. Mix well immediately after addition. Allow to stand at room temperature (18-26°C) for 30 minutes.

7) Transfer contents of tubes to cuvets and read absorbances using blank as reference at the same wavelength and on the same instrument used to prepare the calibration curve. Multiply by the dilution factor to obtain protein concentration in the test sample.

APPENDIX D

Histology

Mallory's consists of several different dyes each of which stains orthochromatically, e.g., the stained tissue element has the same color as the dye; the aniline blue (acidic) stains connective tissue and cartilage; the orange G (acidic) stains blood cells, myelin and muscle; and the acid fuchsin (acidic) stains everything else, including, in shaded of red and pink. One can expect the following results after Mallory's:

Nuclei--red

Muscle and some cytoplasmic elements--red to orange

Nervous system--lilac

Collagen--dark blue

Mucus, connective tissue, and hyaline material--blue

Chitin--red

Myelin and red blood cells--yellow and orange

Dense cellular tissue (liver)--pink with red nuclei

Bone matrix--red

The solutions needed for staining with Mallory's are;

1) Phosphomolybdic acid: phosphomolybdic acid 1.0 gm

dH_2O 100.0 ml

2) Mallory I: acid fuchsin 1.0 gm

dH_2O 100.0 ml

3) Mallory II: Solution A - aniline blue 2.0 gm

dH_2O	100.0 ml
Solution B - orange G.	1.0 gm
dH_2O	100.0ml
Solution C - phosphomolybdic acid	1.0 gm
dH_2O	100.0 ml

To prepare Mallory II staining solution, mix equal quantities of solution A, B, and C prior to using.

The staining procedure to follow is as follow;

1) Xylene--3 min.
2) Xylene--3 min.
3) 100% ETOH--2 min.
4) 95% ETOH--2 min.
5) 70% ETOH--2 min.
6) dH_2O--3 min.
7) Mallory's I--2 min.
8) dH_2O--10 - 30 sec. (differentiates the reds)
9) Phosphomolybdic acid--2 min.
10) dH_2O--dip slide once or twice but no more, purpose of this rinse is to remove excess acid from slide but not from tissue.
11) Mallory's II--10 min.
12) dH_2O-- dip slide in and out until no more color comes off.
13) 0.5% acetic acid--5 min. (produces more transparent sections without altering color).
14) 70% ETOH-- 30 sec.

15) 95% ETOH--30 sec.

16) 100% ETOH--3 min.

17) Xylene--2 min. (mount in coverbond following this step).

APPENDIX E

THE INFLUENCE OF BENZOYL DERIVATIVES ON EARLY DEVELOPMENT IN Tribolium confusum. William E. Oakley, Jr. and Gerhard W. Kalmus.

Eighty-third Annual Meeting of the N.C. Academy of Science, Inc., April 4-5, 1986, at East Carolina University, Greenville, N.C.

Recent discoveries of chitin synthesis inhibitors are leading to the development of more specific insecticidal agents without unduly causing environment damage. Benzoyl peroxide, a bleaching agent in flour, was tested as a possible chitin inhibitor in comparison to a known chitin synthesis inhibitor, diflubenzuron. The confused flour beetle, Tribolium confusum, was utilized as the test model. Sequential developmental stages of T. confusum were exposed to treated flour at concentrations ranging from 0.01 to 100 ppm at 27°C and 60% relative humidity for a thirty day period. The possible insecticidal qualities of benzoyl peroxide were tested against a known chitin synthesis inhibitor, diflubenzuron. Results indicated the following characteristics: egg laying decreased, number of larval stages decreased but the time within each larval stage increased, larval weight decreased, and larval and adult mortality rates increased. These effects were observed using benzoyl peroxide but were more enhanced with diflubenzuron. It is concluded that the utilization of benzoyl peroxide may serve as both a bleaching agent and as an insecticide in commercially produced flour.

CHITIN SYNTHESIS INHIBITION BY BENZOYL DERIVATIVES. William E. Oakley, Jr. and Gerhard W. Kalmus.

Southeastern Regional Developmental Biology Conference, May 1-4, 1986, at Duke University Marine Laboratory, Beaufort, N.C.

Recent discoveries of chitin synthesis inhibitors are leading to the development of more specific insecticidal agents without unduly causing environment damage. Benzoyl peroxide, a bleaching agent in flour, was tested as a possible chitin inhibitor in comparison to a known chitin synthesis inhibitor, diflubenzuron. The confused flour beetle, Tribolium confusum, was utilized as the test model. Sequential developmental stages of T. confusum were exposed to treated flour at concentrations ranging from 0.01 to 100 ppm at 27°C and 60% relative humidity for a thirty day period. The possible insecticidal qualities of benzoyl peroxide were tested against a known chitin synthesis inhibitor, diflubenzuron. Results indicated the following characteristics: egg laying decreased, number of larval stages decreased but the time within each larval stage increased. These effects were observed using benzoyl peroxide but were more enhanced with diflubenzuron. It is concluded that the utilization of benzoyl peroxide may serve as both a bleaching agent and as an insecticide in commercially produced flour.

COMMON FLOUR BEETLE BY AN OXIDIZING AGENT. William E. Oakley, Jr. and Gerhard W. Kalmus.

National Conference of the American Society of Zoologists, Dec. 26-30, 1986, at the Opryland Hotel, Nashville, Tenn.

Tribolium confusum, the confused flour beetle, was used as a model to test the insecticidal potential of ebnzoyl peroxide, an oxidizing agent used to bleach flour. A known chitin synthesis inhibitor, diflubenzuron, which has a chemical structure similar to benzoyl peroxide, was used as a control. Flour was treated with each benzoyl derivative ranging from 0.01 to 100 ppm and progressive developmental stages of T. confusum were exposed to the treated flour for a thirty day period at 27°C and 60% relative humidity. Results indicated that egg laying decreased, the number of larval stages decreased but the time within each larval stage increased, larval weight decreased, and larval and adult mortality rates increased. Measurements of chitin dry weight showed a drop in overall chitin deposition, Polyacrylamide gel electrophoresis indicated a drop in tial protein concentration in benzoyl peroxide treated beetles. Both treatments had similar effects. Thus, this preliminary date suggests a possible common action in controlling T. confusum by interfering with chitin synthesis.

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PRESERVATIVES - DO YOU KNOW WHAT YOU ARE EATING? William E. Oakley, Jr. and Gerhard W. Kalmus.

Southeastern Regional Developmental Biology Conference, Feb 6-8, 1987, in St. Augustine, Florida.

Over the past few years the use of chemical perservatives in various grain products has drawn much public concern. It is important that these preservatives be investigated to examine the possible carcinogenic, teratologic, or growth inhibiting effects. In this study, we investigated the effects of two oxidizing preservatives, benzoyl peroxide and diflubenzuron, which are incorporated into certain grain products in order to increase their shelf-life. An invertebrate organism, Tribolium confusum, was used as a test model. Sequential developmental stages of T. confusum were exposed to treated flour at concentrations ranging from 0.01 to 100 ppm for a thirty day period. Following exposure, several parameters in the developmental process of the flour beetle were examined in order to observe any toxic effects which might have resulted. The parameters examined were: frcundity, teratological defects in various developmental stages, mortality r rates, changes in the biochemical composition of the organism, such as changes in the total protein or glucose levels, histological changes in the exoskeleton, and changes in chitin dry weight.

DEVELOPMENTAL IMPLICATIONS OF FOOD ADITIVES. William E. Oakley, Jr. and Gerhard W. Kalmus.

The American Medical Association 1987 Eastern Student Research Forum, March 2-6, 1987, in Miami, Florida.

In recent years a public concern has arisen over the excessive use of harmful additives in various food products. From an environmental standpoint, it is essential that these additives be examined in order to investigate possible carcinogenic, teratologic, or growth inhibiting effects. In this study we investigated the effects of two oxidizing additives, benzoyl peroxide and diflubenzuron, which are incorporated into certain grain products in order to increase their shelf-life. An invertebrate organism, Tribolium confusum, was used as a test model. Sequential developmental stages of T. confusum were exposed to treated flour at concentrations ranging from 0.01 to 100 ppm for a thirty day period. Following exposure, several parameters in the developmental process of the flour beetle were examined in order to observe any toxic effects which might have resulted. The parameters examined were: fecundity, teratological defects in the various developmental stages, mortality rates in the larval and adult forms, and changes in the total protein and glucose levels. Results indicated that there were definite changes occurring: fecundity decreased by 78%, mortality rates increased by 60 - 100%, glucose and protein levels decreased by 30%, and abnormalities were observed in the chitinous exoskeletons.

THE MORTALITY RATE OF Tribolium confusum EXPOSED TO BENZOYL DERIVATIVES. William E. Oakley, Jr., James Carr, and Gerhard W. Kalmus

Eighty-fourth Annual Meeting of the N.C. Academy of Science, Inc., March 27-28, 1987, at N.C. State University, Raleigh, N.C.

Beetles in the genus Tribolium are one of the most damaging pests of flour in the United States. Since their discovery as an abundant and damaging pest, many insecicides and chemicals have been used in an attempt to control them. Lack of specificity and associated potential environmental insult have rendered most insecticides unacceptable as a control agent. This study shows the effectiveness of diflubenzuron and benzoyl peroxide as possible insecticides in controlling the flour beetle, Tribolium confusum, by exposing different developmental stages of T. confusum to concentrations ranging from 0.01 to 100 ppm, of the test agents. Diflubenzuron is a known chitin synthesis inhibitor, while benzoyl peroxide is used as a bleaching agent which also seems to alter the biosynthesis of chitin. Data indicated that egg laying decreased, the onset of pupation was delayed, termination of pupation was prolonged, and mortality rates increased, Histological evidence indicated abnormal chitin deposition associated within the cuticle. It seems that benzoyl peroxide could be employed in a dual function, i.e., as a bleaching agent and as an insecticide, thus reducing the necessity of numerous additives in order to achieve pest control in commercially available flour.

EFFECTS OF OXIDIZING AGENTS ON FLOUR BEETLE LARVAE. William E. Oakley, Jr. and Gerhard W. Kalmus.

National Conference of The American Society of Biologists, April 8-11, 1987, at the University of Georgia, Athens, Georgia.

The common flour beetle, Tribolium Confusum, was utilized as an invertebrate model to test the insecticidal potential of benzoyl peroxide, an oxidizing agent used in the preservation of flour. Diflubenzuron, a known chitin synthesis inhibitor, which has a chemical composition similar to benzoyl peroxide, was used as our control. Progressive developmental stages of T. confusum were exposed to flour which was previously treated with a series of chemical concentrations ranging from 0,01 to 100ppm. Results indicated that fecundity decreased, larval weight decreased, mortality rates increased. A chitin dry weight measurement showed a drop in overall chitin deposition. Electrophoresis indicated a drop in total protein concentration in treated larvae. Total protein and glucose essays showed decreases and histological examination also indicated small quantities of chitin were being deposited in the exoskeletons. Both treatments had similar effects. Thus, this data indicares that oxidizing agents used in flour do have adverse effects on the development of flour beetle larvae.