AN ARTIFICIAL LARVAL MEDIUM FOR COLONIZED *CULICOIDES GUTTIPENNIS* (COQUILLETT) (DIPTERA: CERATOPOGONIDAE)

by

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Frontispiece. Typical breeding habitat of Culicoides guttipennis.

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I. INTRODUCTION

Although there has been increased interest in Culicoides sp. as vectors of disease, basic studies have been hindered by the inability to rear them in the laboratory. Standardized mass-reared Culicoides colonies are needed for research in the bionomics, nutrition, physiology, disease-vector relationships, and control studies of Culicoides.

According to Linley (1972), there is relatively little known about the nature of food consumed by ceratopogonid larvae. Practically nothing is known about when, and in what relative quantities, the food is normally consumed in relation to the behavior of the larvae. This lack of knowledge of the nutritional requirements of ceratopogonid larvae has, as much as any other factor, held the development of laboratory culture techniques to their present inadequate levels.

The present study was undertaken to develop an artificial larval medium for colonozed Culicoides guttipennis. An artificial larval medium was sought that would provide a uniform condition for larval growth insuring at least a 50% survival rate of the developing larvae. This medium should also incorporate simple rearing techniques, produce large numbers of adults over a relatively short period of time, and provide a medium free from contamination by parasites and predators.

Emphasis in the development of a larval medium was placed on culturing reproducible populations of various microorganisms on which the larvae feed.

II. LITERATURE REVIEW

A. Laboratory Rearing and Colonization Procedures.

The use of laboratory techniques in early studies of Ceratopogonidae consisted mainly of collecting immature forms from their natural breeding sites and allowing them to emerge as adults. Table I lists several ceratopogonids reared from immature stages to adults for various taxonomic or biological studies. Most of the larval diets were substrate collected from natural breeding sites.

Downes (1950) reported the first successful colonization of a ceratopogonid. In his paper Downes simply stated that Culicoides nubeculosus was being maintained in the laboratory. In a report on artificial means of blood feeding, Roberts (1950) referred to the same colony but did not elaborate on colonization procedures. Megahead (1956) took over this colony in 1951 and gave credit to Downes for its establishment at the University of Glasgow in 1947. Megahead stated "the basis of his (Downes) method was to simulate the natural habitat of the species." Soil, rich in manure, was collected by Downes, and placed in earthenware pots with water for larval rearing. It was noted that an algal growth formed on the surface of the substrate. Megahead reared the larvae on a simulated natural diet requiring dried, powdered soil from the original source, together with yeast and powdered charcoal. The colony was maintained until 1953 at which time it began to show symptoms of deterioration due to reduction in the number of eggs hatched.

Table I. A review of some Ceratopogonidae reared in the laboratory to the adult stage.

Species	Larval Diets (Simplified)	Purposes	Reference
Culicoides canithorax and *C. dovei*	Decaying grass roots, humus, brackish water	Life-history and biological studies	Dove, *et al.* (1932)
C. nubeculosus	Horse manure, tap water	Study of *Onchocera cervicalis* development	Steward (1933)
C. impunctatus	Peaty soil	Life-history and biological studies	Hill (1947)
C. obsoletus	Decaying leaves and mud	Life-history and biological studies	Hill (1947)
Leptoconops torrens	Organic detritus and associated microbes	Taxonomy and biological studies	Smith and Lovia (1948)
Culicoides sp.	Soil	Taxonomy	Kettle and Lawson (1952)
C. furens	Bay water, sand, muck	Biological studies	Woke (1954)
C. circumscriptus	Mud, yeast, charcoal, water	Larval behavior and life-history studies	Becker (1958, 1961)
C. loughnani	Rot fluid from rotting stems of prickly pear plants	Biological studies	Jones (1962), Dyce (1969)
Culicoides sp.	Material from natural habitat	Taxonomy	Jamnback and Wirth (1963)

Table I. (Continued)

Species	Larval Diets (Simplified)	Purposes	Reference
Forcipomyia sp., *F. eques*	Decaying wood covered with algae and moss	Taxonomy	Chan and LeRoux (1965)
F. anabaenae	Alga *Anabaena* sp.	Taxonomy	Chan and Saunders (1965)
F. taiwana	Clay (laterite), yeast powder	Life cycle studies	Sun (1965, 1967)
C. mackerrasi, and *C. waringi*	Floating algal mats in water	Autogeny and behavior studies	Dyce and Murray (1967)
Culicoides sp., and *Leptoconops* sp.	Mud, yeast, charcoal, water	Larval biology	Glukhova (1967)
C. bambusicola	Tree-hole water and debris, and blacksoil, mouse pellets, yeast, water	Parthenogenesis and autogeny studies	Lee (1968)
Culicoides sp.	Samples from natural breeding sites	Taxonomy	Battle (1970)
Culicoides sp.	Samples from natural breeding sites	Biological studies	Gazeau and Messersmith (1970)
C. arboricola	Decaying leaves and water	Taxonomy	Linley (1970)
Leptoconops torrens	Soil	Biological studies	Whitsel and Schoeppner (1970)

Table I. (Continued)

Species	Larval Diets (Simplified)	Purposes	Reference
Culicoides sp.	Fleishman's yeast, water	Biological studies	W. W. Smith (1972, Personal communication)
C. arboricola and *C. debilipalpis*	Yeast, nematodes (*Panagrolaimus* sp.), also reared on various commercial fish and turtle food products	Biological studies	W. W. Smith (1972, Personal communication)

The first highly successful colonization was reported by Jones (1957) with C. variipennis sonorensis. He used a larval medium consisting of fresh cow manure and soil combined with enough water to form a thick mud. The complete life cycle in the laboratory was about 30 days, and there was a daily adult emergence of approximately 100 flies. In a later paper, Jones (1960) presented a routine procedure for the propagation of 1,000 adults per day and stated that viable egg production had exceeded the number required to insure colony preservation. The colonization equipment and the procedures used for handling adults was adequate for considerable colony expansion. Jones (1964, 1966) described mass production methods for C. variipennis sonorensis. In the 1966 paper he stated that a revised larval medium from the original colony simulated the physical aspect of the natural medium. A small amount of soil was used to provide microorganisms. Vermiculite was used as a substrate, and cow manure was a source of detritus and nourishment for the microorganisms on which the larvae fed. Jones, et al. (1969) described an improved larval medium for the colonized C. variipennis sonorensis. The condition of the colony was made more uniform and adult production increased. The larval medium consisted of an inert substrate and a microorganism-broth system developed from commercially available products.

Hair (1966) successfully colonized C. guttipennis, and Hair and Turner (1966) described the methods of handling and rearing of this colony. The larvae of C. guttipennis were reared on a medium of decaying leaves collected in a hardwood forest. The success of this

colony in obtaining 1,000 adults per day was attributed to the proper illumination, humidity, and temperature to induce mating. Gazeau and Messersmith (1970) was maintained the same species in the laboratory using a larval medium of stump water and dead leaves.

Since 1966, several other colonies of ceratopogonids have been established. Sjogren and Foulk (1967) reared Leptoconops kerteszi larvae using water-saturated sand with vermiculite, and alfalfa leaf meal. They had trouble inducing mating and suggested that larger mating chambers, to allow male swarming, might be the answer to successful colonization of this species. Boorman (1968) reported rearing C. riethi. The adults are autogenous and were fed only on a sugar solution. Linley (1968a, 1969) reported the successful colonization of C. furens. The larvae were reared on sand collected from the breeding site; the food consisted of measured quantities of a small namatode, Anguillula silusiae. Culicoides melleus was also colonized by Linley (1968b). This species was reared on the previously mentioned namatode with oatmeal and vitamin supplements. Colonization of C. arakawae was reported by Morii and Kitoaka (1968). In this colony, mud, powdered charcoal, yeast, and mouse feed were the ingredients for the larval medium. Sun (1969) reported the successful colonization of C. arakawae and C. schultzei. The larval medium for these species consisted of water and yeast-blood agar cake. No materials from natural breeding habitats were used. Khamala (1971) colonized C. cornutus, using the standard methods for rearing C. variipennis. Rees, et al. (1971) reported rearing Leptoconops kerteszi using washed

oolitic sand, alfalfa leaf meal, and ground dog biscuits in water as a larval medium. Sun (1972) colonized Forcipomyia taiwana, rearing the larvae on laterite clay and yeast powder. Recent colonization attempts have been made by Shad-Del (1969) with C. punctixollis, and Campbell (1971) with C. brevitarsis. In both of these attempts difficulty was encountered with mating.

B. Larval Behavior and Feeding Habits.

Perhaps the earliest observation of ceratopogonid larvae was that of the Reverend W. Derham in 1712. He described the life history of a species of Culicoides, the larvae and pupae of which live in water, during a course of 16 sermons given in St. Mary-le-Bow Church in London. These sermons were assembled into a book by Derham (1713) titled "Physioco-theology: or a Demonstration of the Being and Attributes of God from His Works of Creation." Speaking of the midge, Derham stated "It comes from a little slender Eel-like worm, of a dirty white colour, swimming in stagnating waters by a wriggling motion. . ."

Culicoides larvae have a distinctive movement in their breeding substrate. Becker (1961) observed second-, third-, and fourth-instar C. circumscriptus larvae to be very active in their movements, with the first instar being more sluggish. The larvae move over the surface of the substrate mud in a snake-like fashion, bending the body into a bow, first to one side then the other. These movements were estimated by Becker to take place at a rate of about 550 per minute.

Hill (1947) noted that first stage larvae of C. impunctatus and C. obsoletus were also more sluggish than subsequent instars.

Ceratopogonid larvae have definite behavior patterns in their particular substrates. Continuous searching, presumably for food, is a typical habit. On observations of Central American cerotopogonids, Woke (1954) noted that larvae were seen constantly going under bottom debris. Dyce and Murray (1967) reported that the larvae of C. waringi and C. mackerrasi spent most of the time actively working through the uppermost layer of bottom sludge. Other species seem to prefer surface areas of their substrates as Megahead (1956) observed with C. nubeculosus larvae. The activity of these larvae was facilitated by a free water surface. They were most commonly seen continuously feeding at or below the substrate surface. Becker (1958) maintained that larvae of the majority of species of Culicoides spend most of the time burrowing in mud; but when the necessity arises, they become active swimmers and may occasionally be seen swimming in their natural breeding substrates.

Becker (1957, 1958) conducted an interesting study with C. circumscriptus larvae. He showed that when the larvae were given an adequate diet, the majority exhibited a negative phototaxis. When given an inadequate diet, many exhibited positive phototaxis. His experiments showed that the behavior of C. circumscriptus larvae towards light stimuli is greatly affected by hunger. Becker stated that this method of determining food requirements may be applied to a number of species, depending on their habits and environment. This

could help in answering some questions concerning the typical larval behavioral patterns as previously described. The phototactic studies could also be applied to nutritional studies of several species of ceratopogonids.

Ceratopogonid larvae have varied feeding habits. Some larvae are predaceous, others are primarily herbaceous, while others feed on several kinds of microorganisms such as bacteria, fungi, algae, and Protozoa. Bradley (1954) reported several species of Culicoides larvae feeding on decaying vegetation, protozoans, algae, bacteria, and other microorganisms. Some larvae were strictly carnivorous and cannibalistic. Downes (1958) observed that several species of Culicoides larvae appeared to be unselective in their feeding. Examining their gut contents, he found a mixture of organic fragments and soil particles. Megahead (1956) observed that the larvae of C. nubeculosus feed with little discrimination on a considerable variety of living and non-living organic matter. The culture medium used in rearing this species consisted of amorphous organic matter, algal and fungal growths, sporangia, bacterial film and infusions of ciliates and flagellates. Smith and Lovia (1948) commented on the unselective nature of the food of the larvae of Leptoconops torrens. The normal food of this species consisted of organic detritus with associated microorganisms. Becker (1958) said that the larvae of C. circumscriptus appear to be primarily detritus feeders, feeding mainly on vegetable-matter, both living and non-living. He believed that these larvae were definitely selective in their feeding, observing that they nibble in one spot, leave it and

go elsewhere, constantly changing their position as if searching for particular types of food.

Clarke and Fukuda (1967) reported the larvae of C. cavaticus attacking and killing mosquito larvae in tree-hole water. The ceratopogonid larvae wrapped their bodies around the prey to give the necessary leverage for application of pressure to the mosquitoes. The larvae then penetrated the cuticle of the mosquito larvae, probing beneath it to feed on living tissues. One mosquito was seen to be attacked by several larvae at once. It was also noted that an injured mosquito larva was especially attractive to these larvae. The predaceous habit in this observation was in tree-hole water free from organic debris. When alfalfa pellets were placed into the water, predation ceased. The authors theorized that this behavior of the C. caviticus larvae may have been due to crowding and/or starvation, and that predation was not the true feeding habit of these larvae. Carter (1919) reported Forcipomyia larvae attacking larvae of Aedes aegypti. Kwan and Morrison (1973) observed larvae of C. sanguisuga feeding on dead mites and nematodes. Linley (1968a, 1968b, 1969) fed the nematode Anguilla silusiae to colonized C. furens and C. melleus larvae. W. W. Smith (1972, Personal communication) used Panogrolaimus sp. to rear C. arboricola and C. debilipalpis.

Many investigators have noted herbaceous feeding habits of ceratopogonid larvae. R. Lubega (1972, Personal communication) observed the larvae of C. cornutus feeding on decaying grass particles. He stated that the larvae were not predaceous and did not prey on each

other, even under crowded conditions. Dove, et al. (1932) were able to rear larvae of C. canithorax and C. dovei on decaying grass-roots and humus. Chan and Saunders (1965) reared Forcipomyia anabaenae on algae (Anabaena sp.). They stated that the larvae cannot survive in the absence of Anabaena sp. Algae were reported as a constituent of the food of other ceratopogonid species by Leathers (1923), Bequaert (1925), Painter (1927), Lang (1931), Steward (1933), Mayer (1934), Sequy (1950), Becker (1961), Linley (1971), and Laurence and Mathias (1972).

Other investigations of ceratopogonid larvae feeding on microorganisms include those by Painter (1927) and Laurence and Mathias (1972). Painter found the alimentary canal of C. phlebotomus to be crowded with diatoms. He concluded that diatoms probably constitute the main food of this species. Laurence and Mathias made gut examinations of Leptoconops spinosofrons larvae and determined that these larvae feed by browsing through organic matter ingesting various bacteria, algae, and molds.

Various investigators have incorporated microorganisms into standard techniques for rearing larvae. Saunders (1959) inoculated agar plates with scrapings from rotting leaves to get cultures of several kinds of microorganisms for rearing tree-hole breeding Forcipomyia sp. Lee (1968) used soil in a medium of mouse pellets and yeast to culture bacteria and fungi for rearing C. bambusicola larvae. Jones (1966) described the culture medium of his colony of C. variipennis larvae as providing a suitable medium for microorganism growth.

He noted that since algae and fungi were not available, the larvae fed mainly on bacteria and other small microorganisms. Jones, et al. (1969) later improved the larval medium for the C. variipennis colony by using a mixture of alfalfa meal, high protein supplement, egg albumen, and brain-heart infusion instead of cow manure. This improved artificial system provided for controlled microbial growth.

Linley (1971) reared C. melleus larvae on the nematode, Anguillula silusiae. He was concerned with deficiencies in the larval diet using these nematodes. He compared the suitability of the nematode diet with the natural breeding site (beach sand) in rearing C. melleus. Linley reared adults at the breeding site in half the time taken on the nematode diet. Close examination of the beach sand revealed enormous numbers of protozoans and algae to be present. Linley and Adams (1972) observed that the microorganisms present in the sand constituted the larval food and they were extremely abundant in the area occupied by C. melleus larvae. The problem then was to culture a reproducible algal and protozoan broth in a suitable substrate for the colonized C. melleus.

C. Observations on and Studies with Culicoides guttipennis.

Culicoides guttipennis was described by Coquillett in 1901. Its distribution includes the eastern U.S. from Minnesota to New York, and Oklahoma and Florida. Many researchers have reported host preferences of C. guttipennis. A more recent study by Hair and Turner (1968) indicated that adults of C. guttipennis were general feeders with a

possible preference for large mammals. Humphreys and Turner (1973) collected C. guttipennis from a wide range of avian and mammalian hosts.

Culicoides guttipennis breeds in tree- and stump-holes. Pratt (1907) was probably the first to report this when he found C. guttipennis along with mosquito larvae in dirty poplar stumps. Hair, et al. (1966) found the larvae to be the most abundant tree- and stump-hole breeder in Virginia. They determined that this species could survive in this particular habitat under the following environmental conditions: a temperature range of 2.7 to 30.4°F; a pH of 5.0-8.2; and a dissolved O_2 level of 7.0-10.0 ppm. These authors found the midge in both wet and dry tree- and stump-holes, and comprising over 95% of all collected Culicoides fauna. In addition, they found this species breeding almost continuously during the summer months.

Snow (1949) reported the complete life cycle of C. guttipennis in tree-holes in Illinois. According to Snow, the winter is passed as larvae in the organic ooze at the bottom of the tree-hole. Pupation occurs in April and May. The pupal stage lasts about three days, and the first adults appear in April. The eggs from the first generation are laid on the moist wall of the tree-hole during May and June. The larval stage of the second generation is completed in about three months, and pupation occurs in August and September. Snow collected adults of the second generation as late as October. The eggs of this generation are deposited in September. The larvae hatching from these eggs pass the winter usually in the third instar.

Observations have been made on the larval behavior and feeding habits of C. guttipennis. Pratt (1907) reported them feeding on dead mosquitoes and other insect larvae. In one instance they had accomplished complete disintegration of a syrphid larva, and in another instance rendered the skin of a beetle larva transparent. Foote and Pratt (1954) also reported on C. guttipennis larval feeding. The larvae ingested only decaying vegetation, protozoans, algae, and other microorganisms. Most of the time the larvae remained in the debris at the bottom of the rearing jars. Coher, et al. (1955) added that the larvae burrowed head first in bottom organic material, worked around, emerged partially head first, and undulated like Tubefex.

Hair (1966), and Hair and Turner (1966) reported the successful colonization of C. guttipennis in the laboratory. From this colonization study, field and laboratory observations were made on larval habits. The authors found that the larvae could be scavengers feeding on dead earthworms, dead insects or other organisms as well as organic matter. They were also predators, feeding on live larvae of Orthopodomyia signifera, Aedes triseriatus, and larvae of Helodidae (Coleoptera). Under extremely crowded conditions they were cannibalistic. The larvae were also observed swimming slowly through algal and bacterial mats. The authors maintained that with the existing rearing procedures, the food available for the larvae in the laboratory was limited to bacterial and algal growths, a few small stray invertebrate organisms in the leaf-matter and the leaf-matter itself. In another colony of this same species, Gazeau and Messersmith (1970),

observed that the stump water used for larval rearing contained numerous rotifers, Protozoa, and nematodes (Rhabditis sp.).

Contents from stump-holes in which C. guttipennis typically breed consist of rich organic mud with dark brown water heavily infused in tanins. Many accumulated sediments of decaying leaves, bark, insect fragments, and other such material are usually combined into the organic mud. The contents range from a thick pasty mud, a rich soup, to conditions of being very liquid. Similar descriptions of tree- and stump-hole contents have been reported by Dyce and Murray (1967), Kitching (1971), Park et al. (1950) and Snow (1949, 1958). Stump-hole contents also contain a diverse community of living organisms ranging from many forms of bacteria, fungi, algae, protozoans, platyhelminths, gastrotrichs, nematodes, oligochaetes, several crustaceans and Hydracarina, and a whole array of insects, especially the larval forms of Ceratopogonidae, Psychodidae, Syrphidae, Sarcophagidae, other Diptera, and some Coleoptera. Some significant studies made on the identification of the fauna of tree- and stump-holes include those of Jenkins and Carpenter (1946), Kitching (1969, 1971), Lackey (1939), Maguire (1971), Park et al. (1950), Snow (1949, 1958), and Wirth and Blanton (1967).

E. C. Turner, Jr. has accumulated some unpublished data on the larval diet and media for the C. guttipennis colony. He found that in 1967 difficulties were experienced with the colony when the decayed moist leaves used in larval rearing were allowed to dry in storage. Although water was added back to these leaves, a tremendous reduction

in adult emergence occurred. Examination of this medium showed practically no Ciliata present even several weeks after the water had been added. After switching to moist decayed leaves, the adult production slowly began to rise. During this time of near destruction of the colony, an experiment was made in which fourth instar larvae were placed in water containing certain media and organisms in culture. Table II shows the results of this experiment. The highest percentage adult emergence was from the Paramecium cultures and the natural media. In another experiment, mosquito larvae were placed into a larval rearing chamber containing fourth instar C. guttipennis. The mosquitoes were attacked, vigorously torn apart, and eaten.

Table II. Feeding experiment using fourth instar C. guttipennis.

Diet	Percent Adults Emerged
Paramecium caudatum	83
Natural media	77
Paramecium sp.	73
Fresh house fly body	73
Rotifers	67
Amoeba sp.	63
Distilled water	57
House fly eggs	47
Nematodes (several spp.)	40

III. METHODS AND MATERIALS

A. Condition of the Established Culicoides guttipennis Colony

1. Existing rearing procedure and equipment.

Since the establishment of the Culicoides guttipennis laboratory colony, the rearing procedure, as reported by Hair and Turner (1966), has had few changes. However, for convenience glass food jars (No. 10), painted black to exclude light, were used to rear the larvae instead of the aquaria as originally used. Pint cardboard cartons were placed on top of these jars to collect emerging adults (Figure 1). One-half gallon of decaying leaf-matter and distilled water in a 1:1 ratio were added to the jars. The cardboard cartons easily fit over the mouth of the jars.

The colony was maintained in a walk-in, climatically controlled, rearing room. The temperature was maintained at about 80°F and the relative humidity at about 85%. Lighting in the rearing room consisted of a sun lamp operating on a 13-hour day, and a fluorescent light operating 24 hours. An air conditioner provided an even airflow in the room.

2. Egg viability.

The percentage of viable eggs laid by C. guttipennis females was determined. In this study viable eggs were defined as eggs either having hatched or having anterior eye spots four days after being laid. Eye spots develop in the eggs about 20 hours before hatching. Hatching

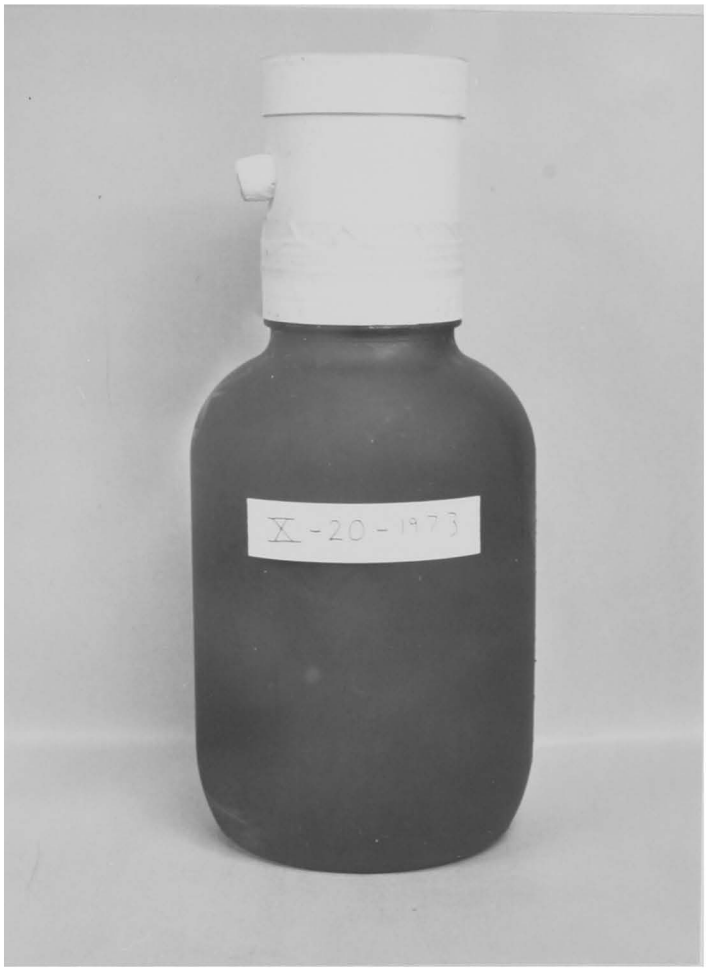

Figure 1. Glass jar rearing container for C. guttipennis larvae.

normally occurs in 3-4 days.

Egg vials were removed daily from the adult holding cages and immediately placed in an egg incubator. After 4 days, the filter paper in the egg vials was removed. The eggs attached to the paper were observed under a binocular microscope, and the viable and non-viable eggs counted. This procedure was continued daily until 10,000 eggs were counted.

3. Adult emergence.

Daily counts of adults emerging from each larval rearing container were recorded for a period of two years (November, 1971 to October, 1973). A new container was started each week and kept for 3-4 months or until no more adults emerged.

Various findings of adult emergence were determined from the daily emergence counts. They were: the mean time from the setting up of a rearing container to the first adult emergence; the mean total adult emergence time from the first to the last adult to emerge; the mean 50% emergence time which is the time from the first adult emergence to the time taken for half the adults to emerge; the monthly total adult emergence; and mean daily adult emergence of the combined larval rearing containers.

B. Determination of C. guttipennis Larval Instars.

A method was developed for determining the instar of individual C. guttipennis larvae for various elapsed time periods after egg hatch.

Kettle and Lawson (1952), and Lawson (1951) used Dyar's law in determining the four instars of various Culicoides larvae. In these two studies three measurements were made of the larval head in determining each instar. Twenty larvae of each instar (mounted on slides) were measured. The three head measurements found to be significantly different for each of the four instars were: (1) head length as measured along the mid-dorsal line, from the hind border of the post-occipital ridge to the front edge of the translucent labrum; (2) head breadth as measured at the widest part of the head; and (3) the width of the oral ring as measured along the hind border of the subgenal band. These head measurements were used in determining the four larval instars in C. guttipennis for subsequent rearing studies.

C. Comparison between Sterile and Non-sterile Larval Media in Rearing C. guttipennis Larvae.

Observations on the larval feeding habits of C. guttipennis by Pratt (1907), Foote and Pratt (1954), Hair (1966), Hair and Turner (1966), and E. C. Turner, Jr. (unpublished data) suggest that this species is at least dependent on some forms of living organisms for its nutritional needs. To verify this, an experiment was set up in which larvae were reared in sterilized decaying leaves. They were compared to larvae reared in non-sterilized decaying leaves containing numerous living organisms. Larval developmental rates, survival rates, and adult emergence were compared.

Decaying leaves, collected in a hardwood forest floor, were soaked in tap water for several weeks and then filtered through cheese cloth.

Equal parts of the solid leaf-matter and its filtered liquid were placed in a food blender and blended at the liquefy setting for 30 seconds. Two parts of the freshly blended mixture were then added to one part of the remaining filtered liquid. The resulting mixture had the same proportion of decaying leaves and liquid as used in the routine larval rearing containers of the established laboratory colony. The purpose in liquefying the decaying leaves, and filtering the liquid, was to insure that no macro-organisms would be present in the final mixture. Wild Culicoides larvae or other arthropods could have been present and possibly would have affected the experimental results of the subsequent experiment. In addition, liquefication of the leaf-matter broke up the organic matter and made nutrients more available for the C. guttipennis larvae.

Part of the blended mixture was placed into 500 ml Erlenmeyer flasks. The flasks were plugged with cotton and placed into a pre-heated steam sterilizer (Electric Steroclave, no. 25X, Wisconsin Alumninum Foundry Co., Manitowoc, Wisconsin) and sterilized at 121°C for 15-20 minutes at 16 lbs pressure. According to the American Public Health Association (1971), the maximum elapsed time for exposure of any nutrient material to heat (from the time of closing the loaded sterilizer to unloading) is 45 minutes. Beyond this time decomposition of sugars occurs due to prolonged exposure to heat. Preheating the sterilizer before loading reduces the total needed heating time to within the 45 minute limit.

Four 20 ml portions of both the sterilized and non-sterilized mixtures of the leaf-matter were placed into 50-dram plastic vials. Twenty viable eggs were then carefully placed in each vial on the mixture surface (eggs of C. guttipennis float readily on a substrate surface without affecting hatching). Each vial was covered with organdy cloth. Records were kept on the number of adults that emerged from each vial.

Daily larval growth and survival rate was also compared. Porcelain plates with 12 one ml cavities were used. Sixty individual cavities were used for each of the sterilized and non-sterilized mixtures. Each cavity was filled with one ml of the appropriate leaf-matter mixture and two viable eggs. The porcelain plates were then placed in enamel pans covered with Saran Wrap to retard evaporation (Figure 2). Each day for 30 days the contents of two cavities were removed from each leaf-matter mixture. Larvae present were removed, and preserved in 70% ethanol. The larval instar was determined for each larva and a record was kept of the daily larval developmental and survival rates for each mixture.

The surface of the C. guttipennis eggs were not sterilized. No suitable sterilizing technique had been developed for them. The eggs were rinsed with distilled water to remove any debris or larger protozoans that may have been clinging to the surface. Beakers, vials, porcelain plates, and instruments used were washed in hot water and alcohol rinses before use. These rinses were not sufficient for complete sterilization. For the purpose of the experiment, though,

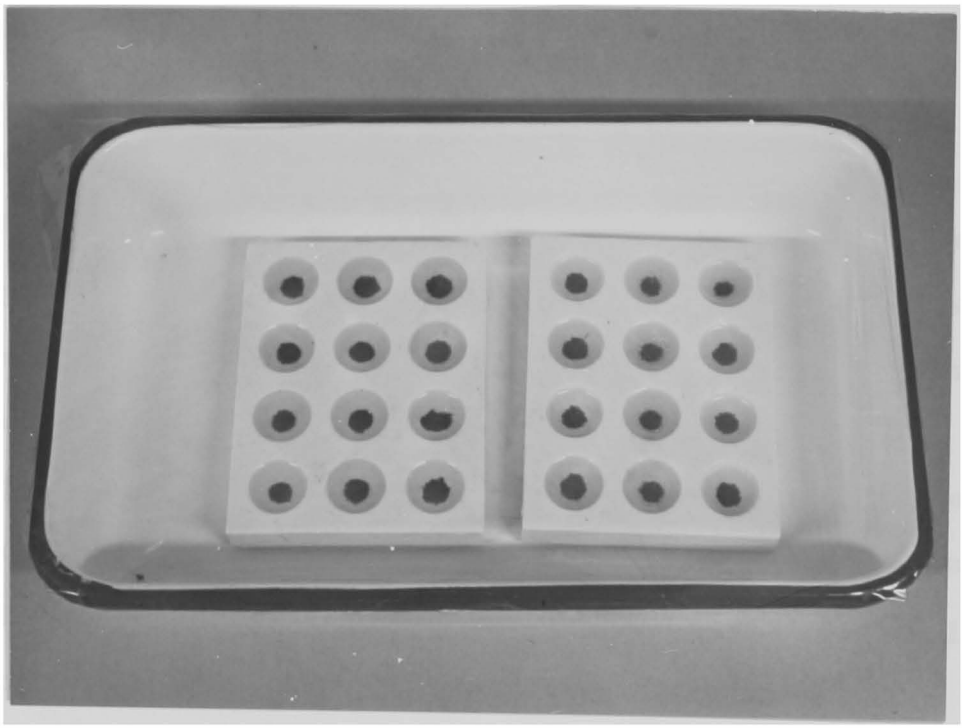

Figure 2. Porcelain plates for rearing larvae of C. guttipennis in sterile and non-sterile leaf-matter for measuring daily larval growth and survival rate.

sterilization other than for the decaying leaf-matter was not necessary. Daily observations of the sterile leaf-matter, in the vials and porcelain plates, were made with a compound microscope. No living organisms were seen for the first week. Some bacteria were obviously present, but in very small numbers. For subsequent experiments, the egg washes and hot water and alcohol rinses were used to help reduce possible sources of contamination.

D. Survey of Protozoan and Microorganism Cultures.

Water from the stored decaying leaves normally used for rearing C. guttipennis larvae was examined with a microscope. Several species of Protozoa were found to be present. Three genera of the Protozoa found in the water, Paramecium sp. (as P. multimicronucleatum), Colpidium sp. (as C. striatum), and Chilomonas sp., were obtained in in culture from Carolina Biological Supply Company at Burlington, North Carolina. In addition, a culture of Euglena sp. (as E. gracilis) was obtained. Various protozoan culture media were prepared as broths for these four organisms. The culture media were also inoculated with both filtered decaying leaf-matter water and filtered stump-hole water to culture any microorganisms present.

Table III lists the various protozoan media used, their ingredients, and the microorganisms attempted in each medium. With medium #1, the water and wheat seeds were boiled for five minutes, and then held overnight before inoculating. This medium was designed by the Carolina Biological Supply Company (1971) for rearing Paramecium sp.

Table III. Protozoan media used in attempting to culture various microorganisms.

Medium#	Ingredients[a]	Protozoan(s) Used[b]	Reference
1	40 wheat seeds, 1 liter water	Paramecium sp., Chilomonas sp., Colpidium sp.	Carolina Biol. Supply Co. (1971)
2	40 wheat seeds, 35 rice grains, 5 cc dry skim milk, 1 liter water	Paramecium sp., Chilomonas sp., Colpidium sp., Euglena sp.	Carolina Biol. Supply Co. (1971)
3	10 g dog chow[c], 50 g rabbit chow[c], 15 g wheat flour, 10 g powdered liver[d], 10 g powdered brewer's yeast, 5 g $CaCO_3$, 20 gal water	Paramecium sp., Chilomonas sp., Colpidium sp., Euglena sp.	Haeger (1961)
4	1 g hay, 0.1 g flour, 700 cc water	Paramecium sp., Chilomonas sp., Colpidium sp., Euglena sp.	Jones (1930, 1937)
5	10.0 g peptone, 0.5 g NH_4NO_3, 2.0 g $NaC_2H_3O_2$, 1.5 g KH_2NO_3, 0.25 g $MgSO_4$, 1 liter water	Chilomonas sp.	Hall (1937)
6	10 g peptone, 2 g $K_2H_2PO_4$, 1 liter water	Chilomonas sp.	Hall (1937)
7	20 g peptone, 2 g KH_2PO_4, 1 liter water	Colpidium sp.	Hall (1937)

Table III. (Continued)

Medium #	Ingredients[a]	Protozoan(s) Used[b]	Reference
8	5.0 g peptone, 0.003 g NaCl, 0.004 g $MgSO_4$, 0.0013 g KNO_3, 0.0002 g $FeCl_3$, 0.015 g $CaSO_4$, 1 liter water	Paramecium sp., Chilomonas sp., Colpidium sp., Euglena	Hall (1937)
9	0.5 g KNO_3, 2.5 g peptone, 0.5 g KH_2PO_4, 0.1 g $MgSO_4$, 0.1 g NaCl, 2.5 g $NaC_2H_3O_2$, 2.0 g dextrose, 1 liter water	Euglena sp.	Hall (1937)
10	1.0 g NH_4NO_3, 0.2 g KH_2PO_4, 0.2 g $MgSO_4$, 0.2 g KCl, trace of $FeCl_3$, 1 liter water	Euglena sp.	Hall (1937)
11	100 cc modified Kleb's solution[f], 40 rice grains, 900 cc water	Euglena sp.	Brandwein (1937)
12	1 Carolina protozoan pellet[g], 20 wheat seeds, 1 liter water	Paramecium sp., Chilomonas sp., Colpidium sp., Euglena sp.	Carolina Biol. Supply Co. (1971)

[a]Distilled water used for each medium.

[b]Leaf-matter and stump-hole microorganisms used with all media.

[c]From Ralston Purina Co., St. Louis, Missouri.

[d]Liver concentrate, Sigma Chemical Co., St. Louis, Missouri.

Table III. (Continued)

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[e]Mixed 40 mesh hay consisting of equal parts of Tall fescue, Timothy, Orchard grass, Brome grass, Reed canary grass, and Kentucky bluegrass. From Agronomy Dept., Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

[f]Modified Kleb's solution: 0.25 g KNO_3, 0.25 g $MgSO_4$, 0.25 g KH_2PO_4, 1.00 g $Ca(NO_3)_2$, 0.01 g Bacto-tryptophane broth powder, 1 liter distilled water.

[g]From Carolina Biological Supply Co., Burlington, North Carolina. Every 20 pellets contain 5000 USP units of vitamin A (carotene), 0.2 mg of vitamin B_2 (riboflavin), 30 mg of vitamin C (ascorbic acid), 0.8 mg of vitamin E (alpha tocopherol), and 0.8 mg vitamin K (phylloquinone).

The ingredients for medium #2 (designed for Euglena sp. by the Carolina Biological Supply Company, 1971) were also added, boiled for five minutes, and held overnight before inoculating. Media # 5-10 described by Hall (1937), were attempted for their appropriate protozoans in trying to rear bacteria-free cultures of Protozoa. In each medium the ingredients were mixed and held overnight before inoculating. Medium #11 was used by Brandwein (1937) for Euglena sp. Rice grains were boiled for 10 minutes and then added to the other ingredients. The medium was held overnight before inoculation. Medium #12 was based on a commercial protozoan pellet obtained from Carolina Biological Supply Company. The wheat seeds used were boiled for ten minutes and added to the water with the dissolved protozoan pellet. This medium also was held overnight before inoculation.

Five hundred ml of each protozoan medium were placed in clear plastic containers with lids (for cultures using medium #4, 700 ml of medium was used). Two replicates for each medium were made up. Twenty ml of each protozoan and microorganism culture were inoculated into each medium. Records were kept on the weekly concentrations of selected Protozoa in each medium for a total of eight weeks. In the protozoan cultures only the Protozoa specified for each culture were counted. In the leaf-matter and stump-hole cultures Protozoa present initially were counted. These cultures were evaluated in rearing their appropriate selected Protozoa so contaminant Protozoa present in the cultures were not counted at this time. Protozoan concentrations were determined by methods similar to those described by Hall, et al.

(1935), and by the American Public Health Association (1971). Incorporation was made using a Sedgewick-Rafter counting chamber and a Whipple eyepiece micrometer. Cultures showing relatively high concentrations of selected Protozoa after four or five weeks were used for later medium studies of C. guttipennis larvae. Those cultures not capable of establishing suitable concentrations were discarded.

The protozoan media tried, with the exception of media #5-10, represent simple media commonly used in culturing Protozoa. These media were chosen from a wide variety of media having similar ingredients.

E. Evaluation of Acceptable Protozoan and Microorganism Cultures in Rearing C. guttipennis Larvae.

1. Attempts using acceptable protozoan and microorganism cultures in rearing C. guttipennis larvae.

Only the protozoan and microorganism cultures that established suitable concentrations of Protozoa were used in determining their effectiveness as possible media for rearing C. guttipennis larvae. The Protozoa media were made up and inoculated with the appropriate protozoan cultures or strained decaying leaf-matter or stump-hole water. The cultures were left to infuse for one week, and rich growths of Protozoa and microorganisms were obtained. Estimates of the concentration of various selected Protozoa in each culture were recorded at this time. Protozoa were identified with the aid of descriptions listed in "Protozoology" by Kudo (1971).

Twenty ml portions of each culture were placed in 20 plastic vials (50-dram). Into each vial 20 viable C. guttipennis eggs were placed on the medium surface. Each vial was then covered with a strip of organdy cloth (Figure 3).

To compare each culture used for larval rearing, the survival rate and developmental rate of C. guttipennis were determined by the following method. For each culture used, four vials were removed every two weeks for a total of eight weeks. The contents of each of the four vials were examined for the developmental stage of the C. guttipennis present. Any larvae found were removed, counted, and preserved for larval instar determination. Pupae were counted, and adults were counted and sexed. Concentration determinations were made of selected Protozoa present in each vial. After eight weeks, four vials remained for each culture. Daily records of adult emergence were kept for each of them. Adults emerging from any of the remaining vials were sexed and the date of emergence was recorded. Vials for adult emergence were held for three months or until no more adults emerged.

Each week after the initiation of the test, 10 ml of a week-old culture were placed into appropriate vials to replace liquid that had evaporated, and in accordance with Laurence (1964), to maintain the population of microorganisms. The additional media added to each vial was inoculated from the appropriate culture of the previous week. The leaf-matter and stump-hole cultures were inoculated with fresh leaf-matter and stump-hole water previously strained through cheese cloth.

Figure 3. 50-dram plastic vial used for C. guttipennis larval medium studies.

As a check for this larval medium study, decaying leaves were blended in a commercial food blender following the same methods and proportions of solid leaf-matter and water as used in the experiment to compare the sterile and non-sterile media. Contents from a stump-hole were taken and prepared in a similar manner. Both of these media were used in accordance with the previously described cultures. New material was blended fresh each week for the weekly inoculations in the plastic vials. Media (without inoculated Protozoa) were also used in this experiment following the methods previously described. These media were made fresh each week and held one week before adding to the vials to replace that which had evaporated.

2. Comparison between fresh and laboratory-adapted stump-hole microorganisms in medium #4 in rearing C. guttipennis larvae.

In the culture of stump-hole microorganisms in medium #4, many Protozoa, rotifers, and bacteria were found. This culture represented microorganisms that had developed for only one week in an artificial medium. An experiment was set up using medium #4 in which laboratory-adapted microorganisms were compared with fresh stump-hole microorganisms as a larval diet. To insure that the stump-hole microorganisms were adapted to the laboratory, they were maintained in the laboratory in medium #4 for four weeks prior to being used as a larval diet. For each of the four weeks after initial inoculation using filtered stump-hole water, new media were prepared and inoculated with 20 ml of the previous week's culture. Only reproducing laboratory

strains of microorganisms were present at the end of four weeks. Twenty 50-dram vials were prepared from this medium using the same methods as employed for the previously described attempts of rearing of C. guttipennis larvae using the acceptable protozoan and microorganism cultures.

3. Incorporation of an artificial substrate in larval medium #4.

After determining the most successful larval medium of fresh stump-hole microorganisms in medium #4, an attempt was made to incorporate it with an artificial substrate in the form of vermiculite to see if it was of additional benefit in rearing C. guttipennis larvae. Vermiculite has good absorptive properties and may possibly absorb the toxic excrement material from the developing larvae and of the microorganisms which settle to the bottom of the rearing container.

In this experiment, fresh medium #4 was prepared and inoculated with 20 ml of strained stump-hole water. One week after infusion, 20 ml of the culture were placed into each of 20 plastic vials (50-dram) as previously described. Next, 10 ml of pre-soaked, clean 20-mesh vermiculite (Terra-lite, No. 3 grade, Construction Products Division, W. R. Grace and Company, Cambridge, Massachusetts) was placed into each vial. When the vermiculite settled to the bottom, 20 viable eggs were placed in each vial. The same methods were applied with these vials as described in the two previous experiments with 10 ml of a seven-day-old culture added weekly to each vial. No

additional vermiculite was added. Results obtained from this rearing method using vermiculite were compared to the results using the same culture without the vermiculite.

IV. RESULTS

A. Condition of the Established C. guttipennis Colony.

1. Egg viability.

Of 10,000 eggs laid by colonized C. guttipennis females in the laboratory, 67% were viable. This result is in agreement with Boorman (1973) who found considerable mortality in the egg stage of ceratopogonid colonies. Boorman reported an egg hatch of 70% in colonized C. variipennis and C. riethi, while in C. nubeculosus, the egg hatch was 55%.

2. Adult emergence.

The following are calculations from daily records of larval rearing containers made during the two-year study of the C. guttipennis colony. The mean time elapsed from the start of a rearing container until the emergence of the first adult was 23.0 $\pm$ 6.8 days. The mean total emergence time from that of the first adult until the last adult emerged was 77.4 $\pm$ 41.9 days. The mean emergence time for 50% of the adults to emerge was 25.9 $\pm$ 15.8 days.

The data in Table IV show the total monthly adult emergence and the mean daily adult emergence for each month of the two-year period of all larval rearing containers incorporating the decaying leaves. Figure 4 is a graphical presentation of the mean daily adult emergence for each month in the two-year period. There appeared to be a decrease in adult emergence over the two-year period.

Table IV. Monthly C. guttipennis adult emergence records for two years.

Month	Total Adult Emergence	Mean Daily Emergence (X ± SD)
November, 1971	5,543	184.8 ± 102.6
December, 1971	3,982	128.5 ± 41.4
January, 1972	4,578	147.7 ± 52.0
February, 1972	4,955	170.9 ± 66.2
March, 1972	2,937	94.7 ± 76.3
April, 1972	2,016	67.2 ± 75.7
May, 1972	3,595	116.0 ± 48.3
June, 1972	3,837	127.9 ± 41.0
July, 1972	2,001	64.6 ± 35.9
August, 1972	3,681	118.7 ± 68.0
September, 1972	1,859	62.0 ± 23.4
October, 1972	3,001	96.8 ± 50.8
November, 1972	2,435	81.2 ± 29.5
December, 1972	4.242	136.8 ± 51.6
January, 1973	3,008	97.1 ± 31.0
February, 1973	4,321	154.3 ± 38.5
March, 1973	2,927	94.4 ± 53.3
April, 1973	2,501	83.4 ± 42.5
May, 1973	3,690	119.0 ± 51.4
June, 1973	2,132	71.1 ± 26.9
July, 1973	2,994	96.6 ± 34.3

Table IV. (Continued)

Month	Total Adult Emergence	Mean Daily Emergence ($\bar{X} \pm SD$)
August, 1973	2,036	65.7 ± 17.9
September, 1973	2,372	79.4 ± 28.7
October, 1973	3,298	106.4 ± 37.6

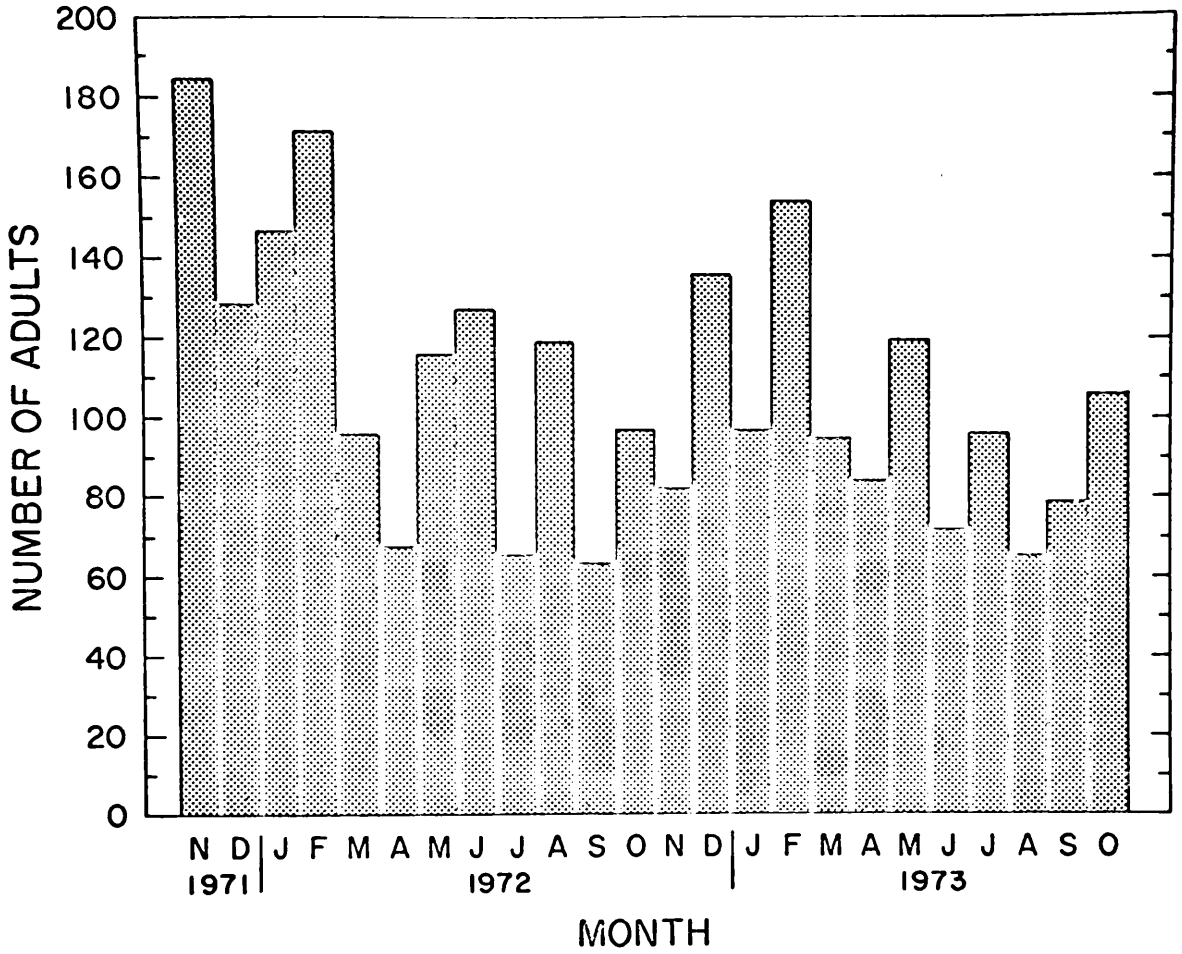

Figure 4. Mean daily adult emergence of C. guttipennis for two years.

B. Determination of C. guttipennis Larval Instars.

Kettle and Lawson (1952) stated that the head size of ceratopogonid larvae is normally distributed and can be represented by the expression $\bar{X}$ + SD where $\bar{X}$ = the mean and SD = the standard deviation of the population of the mean. Since the distribution is normal, two-thirds of the population will have head sizes ranging from $\bar{X}$ - SD to $\bar{X}$ + SD. The data in Table V show the head measurements of each larval instar of C. guttipennis as $\bar{X} \pm$ SD. These values for each instar were used for larval instar determination in later larval rearing studies.

C. Comparison between Sterile and Non-sterile Leaf-Matter in Rearing C. guttipennis Larvae.

The data in Table VI show the number of C. guttipennis adults emerging from both the sterile and non-sterile leaf-matter mixtures. A mean of 10.5 of a possible 20 adults emerged from each of the four vials containing non-sterile leaf-matter.

Table VII presents a comparison of C. guttipennis larval developmental and survival rates for a 30-day period in the sterile and non-sterile leaf-matter. Daily records showed that larvae in the sterile leaf-matter did not develop beyond the first instar, and no larvae were found alive after four days. However, in the non-sterile leaf-matter, larvae were able to develop to the fourth instar with a survival rate of 2.5 larvae per day for the 30-day period. The marked differences in larval development between these two media are easily seen. It is apparent that even inadequate sterile techniques, after

Table V. C. guttipennis larval instar head measurements (millimeters).[a]

Instar	Length ($\bar{X} \pm SD$)	Breadth ($\bar{X} \pm SD$)	Anterior Ring ($\bar{X} \pm SD$)
1	65.6 ± 2.1	40.6 ± 2.3	24.9 ± 2.5
2	99.7 ± 2.0	60.7 ± 2.0	40.1 ± 2.7
3	154.2 ± 3.8	91.6 ± 9.0	65.2 ± 9.0
4	238.8 ± 7.0	158.8 ± 12.3	119.8 ± 13.1

[a]20 larvae of each instar were measured.

Table VI. C. guttipennis adult emergence from sterile and non-sterile leaf-matter.

Vial	Adults from Non-Sterile Leaf-Matter	Adults from Sterile Leaf-Matter
1	12 (8♂, 4♀)	0
2	9 (6♂, 3♀)	0
3	10 (8♂, 2♀)	0
4	11 (6♂, 5♀)	0
Total	42	0
$\bar{X}$	10.5	0

Table VII. C. guttipennis larval survival and developmental rates in sterile and non-sterile leaf-matter over a 30-day period.

Day	Non-sterile Leaf-Matter					Sterile Leaf-Matter				
	Larval Instar				Total	Larval Instar				Total
	1	2	3	4	Larvae	1	2	3	4	Larvae
1	3				3	2				2
2	4				4	3				3
3	2				2	2				2
4	3	1			4	2				2
5	1	2			3					0
6		3	1		4					0
7		1	2		3					0
8		2	1		3					0
9		2	1		3					0
10		2			2					0
11			2		2					0
12			1		1					0
13			2		2					0
14			1		1					0
15			1		1					0
16			2	1	3					0
17		1	1		2					0
18		1	1		2					0
19			2		2					0
20		2	2		4					0
21			3		3					0
22			3		3					0
23			2		2					0
24			3		3					0
25			1		1					0
26			3	1	4					0
27			2		2					0
28			2		2					0
29			4		4					0
30		1		1	2					0
Mean Survival Rate ($\bar{X}$) = 2.5						Mean Survival Rate ($\bar{X}$) = 0.3				

the initial leaf-matter sterilization, had no great influence in altering the results.

D. Survey of Protozoan and Microorganism Cultures.

The counts of selected Protozoa in each protozoan and microorganism culture for a total of eight weeks are shown in Table VIII. Concentrations indicated by a (+) contained more than 300 individual Protozoa per milliliter. Concentrations indicated by a (-) contained less than 300 individual Protozoa per milliliter. A (0) indicates no selected Protozoa were present. Protozoa having (+) concentrations in their appropriate media after three weeks were considered suitable for attempting to use in C. guttipennis larval rearing studies. The suitable protozoan and microorganism cultures include Paramecium sp. in media #3, 4, and 12; Chilomonas sp. in media #3 and 12; Colpidium sp. in media #3 and 4; Euglena sp. in media #2 and 12; leaf-matter Protozoa in media #2, 3, 4, and 12; and stump-hole Protozoa in media #3, 4, and 12. In attempts with bacteria-free cultures using media #5 to 10, no Protozoa except Euglena sp. in medium #9 became established. However, a thick scum formation killed the Euglena after the first week. With the other protozoan and microorganism cultures, good growth occurred during the first or second weeks. The protozoan concentrations dropped readily after that, approaching zero within three or four weeks.

Table VIII. Weekly counts of selected Protozoa in various media.[a]

Protozoa	Media	Replicate	Week 1	2	3	4	5	6	7	8
Paramecium sp.	3	1	+	+	+	-	0	0	0	0
		2	+	+	+	+	-	0	0	0
Paramecium sp.	4	1	+	+	+	+	-	-	0	0
		2	+	+	+	+	+	-	0	0
Paramecium sp.	12	1	+	+	+	+	+	-	-	0
		2	+	+	+	+	0	0	0	0
Chilomonas sp.	3	1	+	+	+	+	+	+	+	-
		2	+	+	+	+	-	-	0	0
Chilomonas sp.	4	1	+	-	-	-	-	0	0	0
		2	+	-	-	0	0	0	0	0
Chilomonas sp.	12	1	+	+	+	+	+	-	-	-
		2	+	+	+	+	+	+	-	-
Colpidium sp.	3	1	+	+	+	-	0	0	0	0
		2	+	+	+	-	-	0	0	0
Colpidium sp.	4	1	+	+	+	+	-	-	0	0
		2	+	+	+	+	+	+	-	-
Colpidium sp.[b]	12	1	+	-	0	0	0	0	0	0
		2	+	0	0	0	0	0	0	0
Euglena sp.	2	1	+	+	+	+	+	+	+	+
		2	+	+	+	+	+	+	+	+
Euglena sp.	3	1	-	-	0	0	0	0	0	0
		2	-	0	0	0	0	0	0	0
Euglena sp.	4	1	+	-	0	0	0	0	0	0
		2	-	-	0	0	0	0	0	0
Euglena sp.[c]	9	1	+	0	0	0	0	0	0	0
		2	+	0	0	0	0	0	0	0
Euglena sp.	12	1	+	+	+	+	+	+	+	+
		2	+	+	+	+	+	+	+	+

Table VIII. (Continued)

Protozoa	Media	Replicate	Week 1	2	3	4	5	6	7	8
Leaf-matter Protozoa[d]	1	1	+	-	0	0	0	0	0	0
		2	+	-	-	0	0	0	0	0
Leaf-matter Protozoa	2	1	+	+	+	+	-	-	-	-
		2	+	+	+	+	+	-	-	-
Leaf-matter Protozoa	3	1	+	+	+	+	+	+	+	+
		2	+	+	+	+	+	+	+	+
Leaf-matter Protozoa	4	1	+	+	+	+	+	+	+	+
		2	+	+	+	+	+	+	+	+
Leaf-matter Protozoa	12	1	+	+	+	+	+	+	+	+
		2	+	+	+	+	+	+	+	+
Stump-hole Protozoa[d]	1	1	+	-	-	0	0	0	0	0
		2	-	-	0	0	0	0	0	0
Stump-hole Protozoa	3	1	+	+	+	+	-	-	-	-
		2	+	+	+	+	+	+	-	-
Stump-hole Protozoa	4	1	+	+	+	+	+	+	+	+
		2	+	+	+	+	+	+	+	+
Stump-hole Protozoa	12	1	+	+	+	+	+	+	+	+
		2	+	+	+	+	+	+	+	+

[a]Cultures not able to establish growths of their appropriate Protozoa include: Paramecium sp. in media #1, 2, and 8; Chilomonas sp. in media #1, 2, 5, 6, and 8; Colpidium sp. in media #1, 2, 7, and 8; Euglena sp. in media #8, 10, and 11; leaf-matter Protozoa in media #5, 6, 7, 8, 9, 10, and 11; and stump-hole Protozoa in media #2, 5, 6, 7, 8, 9, 10, and 11.

[b]After one week Colpidium sp. was overpopulated by a contaminant ciliate Colpoda sp.

[c]There was good growth of Euglena sp. for one week, but a thick scum formation after that time quickly killed off the culture.

[d]In the leaf-matter and stump-hole cultures, Protozoa present initially were counted each week (contaminant organisms were not counted).

E. Evaluation of Acceptable Protozoan and Microorganism Cultures in Rearing C. guttipennis.

1. Attempts using acceptable protozoan and microorganism cultures in rearing C. guttipennis larvae.

After one week of infusion, each of the acceptable cultures had abundant concentrations of Protozoa. The data in Table IX show the estimated concentrations of the selected Protozoa that were counted in each culture. The numbers in parenthesis represent the number of individual organisms per milliliter. Many contaminant organisms were present in each culture. Several small flagellates appeared in all the culture containers. Amoeboid forms and other small organisms were present in most cultures, but were too small or not easily recognized, thus preventing accurate estimates of their concentrations.

Lists of each protozoan culture used for larval rearing and the mean number of surviving individuals in each set of four vials of all larval stages are presented in Table X. The mean total number of surviving individuals of all 20 vials is given for each culture. Each mean presented is in the form of $\bar{X} \pm SD$ where $\bar{X}$ is the mean and SD is standard deviation of the mean.

The results in Table X reveal that a total survival rate and adult emergence of 50% or greater was obtained in only five cultures. The results were then used for further analysis to determine which of these cultures, if any, was superior.

In Table XI the mean estimated concentrations of the dominant forms of Protozoa present in each of the best five cultures at each two-week period of examination is shown. At each two-week interval,

Table IX. Initial protozoan concentrations in acceptable protozoan and microorganism cultures used for C. guttipennis larval medium studies.

Culture	Initial Protozoan Concentrations (mean number of individual organisms per ml)
Paramecium sp. medium #3	Paramecium sp. (1,500); Colpoda sp. (1,000) Flagellates - Bodo sp., Monas sp., Ochromonas sp., Pleuromonas sp. (3,000)
Paramecium sp. medium #4	Paramecium sp. (600); Flagellates - Bodo sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (50,000)
Paramecium sp. medium #12	Paramecium sp. (1,000); Flagellates - Bodo sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (20,000)
Chilomonas sp. medium #3	Chilomonas sp. (3,000); Colpoda sp. (600); Flagellates- Bodo sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (5,000)
Chilomonas sp. medium #12	Chilomonas sp. (1,000); Flagellates - Bodo sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (100,000)
Colpidium sp. medium #3	Colpidium sp. (7,500); Peranema sp. (600); Flagellates- Bodo sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (50,000)
Colpidium sp. medium #4	Colpidium sp. (1,000); Flagellates - Bodo sp., Collodictyon sp. (50,000)
Euglena sp. medium #2	Euglena sp. (200,000); Peranema sp. (1,000)
Euglena sp. medium #12	Euglena sp. (5,000); Flagellates - Bodo sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (100,000)
Leaf-matter Protozoa medium #2	Colpidium sp. (100); Oxytrichidae (200); Flagellates- Bodo sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (75,000)
Leaf-matter Protozoa medium #3	Paramecium sp. (1,000); Oxytrichidae (150); Tetrahymena sp. (1,000); Tracheophyllum sp. (100); Vorticella sp. (150); Rotifers - Philodina sp. (100)

Table IX. (Continued)

Culture	Initial Protozoan Concentrations (mean number of individual organisms per ml)
Leaf-matter Protozoa medium #4	Colpidium sp. (500); Oxytrichidae (200); Paramecium sp. (350); Peranema sp. (250); Flagellates - Bodo sp., Cercomonas sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (100,000); Rotifers - Philodina sp. (150)
Leaf-matter Protozoa medium #12	Colpidium sp. (2,000); Oxytrichidae (300); Paramecium sp. (400); Tetrahymena sp. (300); Chilomonas sp. (5,000); Peramena sp. (250); Flagellates - Bodo sp., Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (10,000)
Stump-hole Protozoa medium #3	Tetrahymena sp. (1,000); Vorticella sp. (300); Astasia sp. and Rhabdomonas sp. (400); Flagellates - Bodo sp., Cercomonas sp., Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (50,000)
Stump-hole Protozoa medium #4	Colpoda sp. (300); Loxodes sp. (350); Microthorax sp. (350); Tetrahymena sp. (350); Vorticella sp. (200); Astasia sp. and Rhabdomonas sp. (3,000); Flagellates- Cercomonas sp., Collodictyon sp. (100,000); Rotifers- Philodina sp. (150)
Stump-hole Protozoa medium #12	Loxodes sp. (400); Microthorax sp. (400); Tetrahymena sp. (350); Vorticella sp. (300); Astasia sp. and Rhabdomonas sp. (400); Flagellates - Bodo sp., Cercomonas sp., Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (40,000)
Blank medium #2	Flagellates - Collodictyon sp. (50,000)
Blank medium #3	Flagellates - Bodo sp., Monas sp., Ochromonas sp., Pleuromonas sp. (2,000)
Blank medium #4	Flagellates - Collodictyon sp. (50,000)
Blank medium #12	Flagellates - Collodictyon sp., Monas sp., Ochromonas sp., Pleuromonas sp. (50,000)

Table IX. (Continued)

Culture	Initial Protozoan Concentrations (mean number of individual organisms per ml)
Blended leaf-matter mixture	Colpidium sp. (1,000); Oxytrichidae (250); Paramecium sp. (300); Tracheophyllum sp. (100); Vorticella sp. (200); Chilomonas sp. (3,000); Peranema sp. (200); Flagellates - Bodo sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (30,000); Rotifers - Philodina sp. (150)
Blended stump-hole mixture	Colpoda sp. (400); Loxodes sp. (2,000); Microthorax sp. (1,000); Tetrahymena sp. (1,000); Vorticella sp. (400); Astasia sp. and Rhabdomonas sp. (30,000); Flagellates-Cercomonas sp., Cyathomonas sp. (30,000); Rotifers-Philodina sp. (150)

Table X. Mean survival rates of C. guttipennis in acceptable protozoan and microorganism cultures.

Culture	2 Weeks ($\bar{X} \pm SD$)	4 Weeks ($\bar{X} \pm SD$)	6 Weeks ($\bar{X} \pm SD$)	8 Weeks ($\bar{X} \pm SD$)	Adults ($\bar{X} \pm SD$)	Total ($\bar{X} \pm SD$)
Stump-hole micro-organisms medium #4	14.8 ± 4.0	14.8 ± 2.1	10.8 ± 2.5	15.0 ± 1.8	14.0 ± 1.4	13.9 ± 2.8
Blended stump-hole mixture	13.3 ± 3.2	15.0 ± 1.4	11.5 ± 3.7	16.5 ± 2.4	13.0 ± 0.8	13.9 ± 2.9
Colpidium sp. medium #4	17.0 ± 1.6	11.3 ± 1.7	12.5 ± 2.9	13.3 ± 1.3	12.0 ± 4.7	13.2 ± 3.2
Blended leaf-matter mixture	12.5 ± 1.9	10.8 ± 2.2	10.8 ± 1.7	11.3 ± 2.1	13.3 ± 2.1	11.7 ± 2.1
Stump-hole micro-organisms medium #12	11.5 ± 4.0	12.0 ± 6.8	11.0 ± 2.9	11.5 ± 3.5	11.5 ± 0.6	11.5 ± 3.7
Colpidium sp. medium #3	15.5 ± 1.7	12.3 ± 2.1	10.0 ± 3.5	5.3 ± 1.3	3.8 ± 3.3	9.4 ± 5.0
Paramecium sp. medium #4	8.8 ± 3.9	11.8 ± 4.6	5.8 ± 4.6	7.3 ± 2.2	8.8 ± 2.5	8.5 ± 4.1
Leaf-matter microorganisms medium #12	16.8 ± 1.0	12.3 ± 2.5	3.5 ± 2.1	4.5 ± 3.0	4.3 ± 2.5	8.3 ± 5.8

Table X. (Continued)

Culture	2 Weeks ($\bar{X} \pm SD$)	4 Weeks ($\bar{X} \pm SD$)	6 Weeks ($\bar{X} \pm SD$)	8 Weeks ($\bar{X} \pm SD$)	Adults ($\bar{X} \pm SD$)	Total ($\bar{X} \pm SD$)
Chilomonas sp. medium #12	10.5 ± 5.8	7.5 ± 5.2	5.8 ± 2.6	10.0 ± 5.3	6.0 ± 4.1	8.0 ± 4.6
Leaf-matter microorganisms medium #4	11.3 ± 2.9	10.8 ± 3.3	7.3 ± 1.3	6.5 ± 2.5	2.0 ± 1.6	7.6 ± 4.1
Paramecium sp. medium #3	7.0 ± 3.6	8.5 ± 3.4	6.8 ± 3.4	5.0 ± 2.2	2.5 ± 1.3	6.0 ± 3.3
Chilomonas sp. medium #3	4.8 ± 1.7	4.3 ± 3.0	1.5 ± 1.3	11.8 ± 2.5	6.5 ± 0.6	5.8 ± 4.0
Blank medium #3	11.8 ± 4.0	7.8 ± 1.7	5.5 ± 3.1	1.3 ± 1.5	0.8 ± 1.5	5.4 ± 4.8
Stump-hole microorganisms medium #3	11.8 ± 2.6	7.0 ± 2.9	3.0 ± 3.6	4.3 ± 5.7	1.3 ± 0.5	5.4 ± 4.7
Leaf-matter microorganisms medium #2	6.8 ± 1.0	4.3 ± 2.2	3.8 ± 5.6	6.0 ± 2.7	4.5 ± 3.3	5.1 ± 3.2
Leaf-matter microorganisms medium #3	5.8 ± 1.3	4.5 ± 2.7	4.5 ± 1.3	5.3 ± 3.0	4.0 ± 2.2	4.8 ± 2.0
Euglena sp. medium #2	4.3 ± 1.7	4.0 ± 0.8	4.0 ± 0.8	3.3 ± 1.5	3.8 ± 5.0	3.9 ± 2.3

Table X. (Continued)

Culture	2 Weeks ($\bar{X} \pm SD$)	4 Weeks ($\bar{X} \pm SD$)	6 Weeks ($\bar{X} \pm SD$)	8 Weeks ($\bar{X} \pm SD$)	Adults ($\bar{X} \pm SD$)	Total ($\bar{X} \pm SD$)
Blank medium #4	4.0 ± 4.2	0.5 ± 0.6	0.8 ± 1.0	4.0 ± 4.8	1.0 ± 0.8	2.6 ± 3.1
Euglena sp. medium #12	3.5 ± 3.5	4.5 ± 3.0	2.8 ± 1.5	0.7 ± 1.0	0.5 ± 0.6	2.4 ± 2.5
Paramecium sp. medium #12	2.5 ± 3.3	3.0 ± 1.6	2.3 ± 2.2	0.3 ± 0.5	0	1.6 ± 2.1
Blank medium #12	0.3 ± 0.5	0.5 ± 1.0	0	0.3 ± 0.5	0	0.2 ± 0.5
Blank medium #2	0	0	0	0	0	0

Table XI. Mean protozoan concentrations for eight weeks of the best five protozoan and microorganism cultures for rearing C. guttipennis larvae.

Culture	Week	Mean Protozoan Concentrations (Mean number of individual organisms per ml)
Stump-hole Protozoa medium #4	2	Microthorax sp. (2,000); Oxytrichidae (150)[a]; Tetrahymena sp. (100)[b]; Tracheophyllum sp. (100)[c]; Vorticella sp. (100); Astasia sp. and Rhabdomonas sp. (20,000); Flagellates - Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (40,000); Rotifers - Philodina sp. (150)
	4	Microthorax sp. (3,000); Oxytrichidae (100)[d]; Vorticella sp. (200); Astasia sp. and Rhabdomonas sp. (45,000); Flagellates - Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (20,000); Rotifers - Philodina sp. (200)
	6	Microthorax sp. (5,000); Vorticella sp. (150); Astasia sp. and Rhabdomonas sp. (70,000); Flagellates - Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (50,000); Rotifers - Philodina sp. (250)
	8	Microthorax sp. (7,500); Vorticella sp. (150) Astasia sp. and Rhabdomonas sp. (75,000); Flagellates - Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (20,000); Rotifers - Philodina sp. (150)
Blended stump-hole mixture	2	Loxodes sp. (350); Microthorax sp. (2,000); Tetrahymena sp. (600); Vorticella sp. (200); Astasia sp. and Rhabdomonas sp. (600); Flagellates - Cercomonas sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (1,600); Rotifers - Philodina sp. (200)
	4	Loxodes sp. (300); Microthorax sp. (1,000); Tracheophyllum sp. (100)[e]; Astasia sp. and Rhabdomonas sp. (300); Peranema sp. (400); Flagellates - Cercomonas sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (1,000); Rotifers- Philodina sp. (300)
	6	Microthorax sp. (1,000); Astasia sp. and Rhabdomonas sp. (1,000); Flagellates - Cercomonas sp., Cythomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (2,000); Rotifers - Philodina sp. (150)

Table XI. (Continued)

Culture	Week	Mean Protozoan Concentrations (Mean number of individual organisms per ml)
	8	Loxodes sp. (100); Microthorax sp. (1,000); Vorticella sp. (100); Astasia sp. and Rhabdomonas sp. (1,000); Flagellates - Cercomonas sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (600); Rotifers - Philodina sp. (100)
Colpidium sp. medium #4	2	Colpidium sp. (300); Microthorax sp. (200); Flagellates - Bodo sp., Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (100,000)
	4	Colpidium sp. (300); Flagellates - Bodo sp., Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (7,500)
	6	Colpidium sp. (300); Flagellates - Cyathomonas sp. (250)
	8	Colpidium sp. (350); Flagellates - Cyathomonas sp. (400)
Blended leaf-matter mixture	2	Cyclidium sp. (400); Oxytrichidae (300); Paramecium sp. (500); Vorticella sp. (500); Peranema sp. (300); Flagellates - Bodo sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (4,000); Rotifers - Philodina sp. (300)
	4	Halteria sp. (200); Paramecium sp. (600); Vorticella sp. (600); Astasia sp. (1,000); Peranema sp. (300); Flagellates - Bodo sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (2,000); Rotifers - Philodina sp. (300)
	6	Colpidium sp. (300); Oxytrichidae (200); Paramecium sp. (400); Vorticella sp. (300); Astasia sp. (300); Peranema sp. (400); Flagellates - Bodo sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (15,000); Rotifers - Philodina sp. (300)
	8	Halteria sp. (150)[f]; Paramecium sp. (300); Tracheophyllum sp. (100)[g]; Vorticella sp. (300); Astasia sp. (300); Chilomonas sp. (200); Peranema sp. (400); Flagellates - Bodo sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (4,000); Rotifers - Philodina sp. (325)

Table XI. (Continued)

Culture	Week	Mean Protozoan Concentrations (Mean number of individual organisms per ml)
Stump-hole Protozoa medium #12	2	Loxodes sp. (300); Microthorax sp. (400); Tetrahymena sp. (300); Vorticella sp. (200); Astasia sp. and Rhabdomonas sp. (400); Flagellates - Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (75,000); Rotifers - Philodina sp. (200)
	4	Loxodes sp. (150); Microthorax sp. (250); Vorticella sp. (200); Rhabdomonas sp. (100); Flagellates - Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (20,000); Rotifers - Philodina sp. (150)
	6	Loxodes sp. (100); Microthorax sp. (200); Vorticella sp. (100); Rhabdomonas sp. (100); Flagellates - Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (1,000); Rotifers - Philodina sp. (250)
	8	Loxodes sp. (100)[h]; Microthorax sp. (250); Vorticella sp. (200)[i]; Flagellates - Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (3,000); Rotifers - Philodina sp. (300)

[a]Oxytrichidae observed in one vial.

[b]Tetrahymena sp. observed in two vials.

[c]Tracheophyllum sp. observed in one vial.

[d]Oxytrichidae observed in one vial.

[e]Tracheophyllum sp. observed in one vial.

[f]Halteria sp. observed in one vial.

[g]Tracheophyllum sp. observed in one vial.

[h]Loxodes sp. observed in one vial.

[i]Vorticella sp. observed in one vial.

an abundance of Protozoa were present in each culture along with many contaminant organisms.

The data in Figure 5 show the total number of surviving individuals of each developmental stage of C. guttipennis for each culture of the four vials combined for each two-week period. A progression from early instars at the first two weeks to almost entirely adults at eight weeks occurred in each culture. In Figure 6, only the adult emergence for each two-week period is presented. There appears to be a linear progression for each culture from the first adult emergence at four weeks through the eighth week. The total adult emergence for each culture is included for comparison. After eight weeks there was a considerable leveling off in the adults emerging. In Figure 6 the numbers of adults for each culture represent the total number of adults that have emerged from each set of four vials combined.

A statistical analysis was made of the adult emergence data of each culture for each of the two-week periods. These data are presented in Table XII. This was a two-factor, factorial experiment in which the cultures were qualitative and the weeks were quantitative. From the data given in Table XII, linear and quadratic week effects using orthogonal coefficients were extracted to see how adult emergence varies in each culture during each two-week period. The data in Table XIII show the results of the analysis of variance. There was a significant difference at the 1% level in the number of adults emerging from each culture at each two-week period. This was a difference in the linear progression of adult emergence also found to be

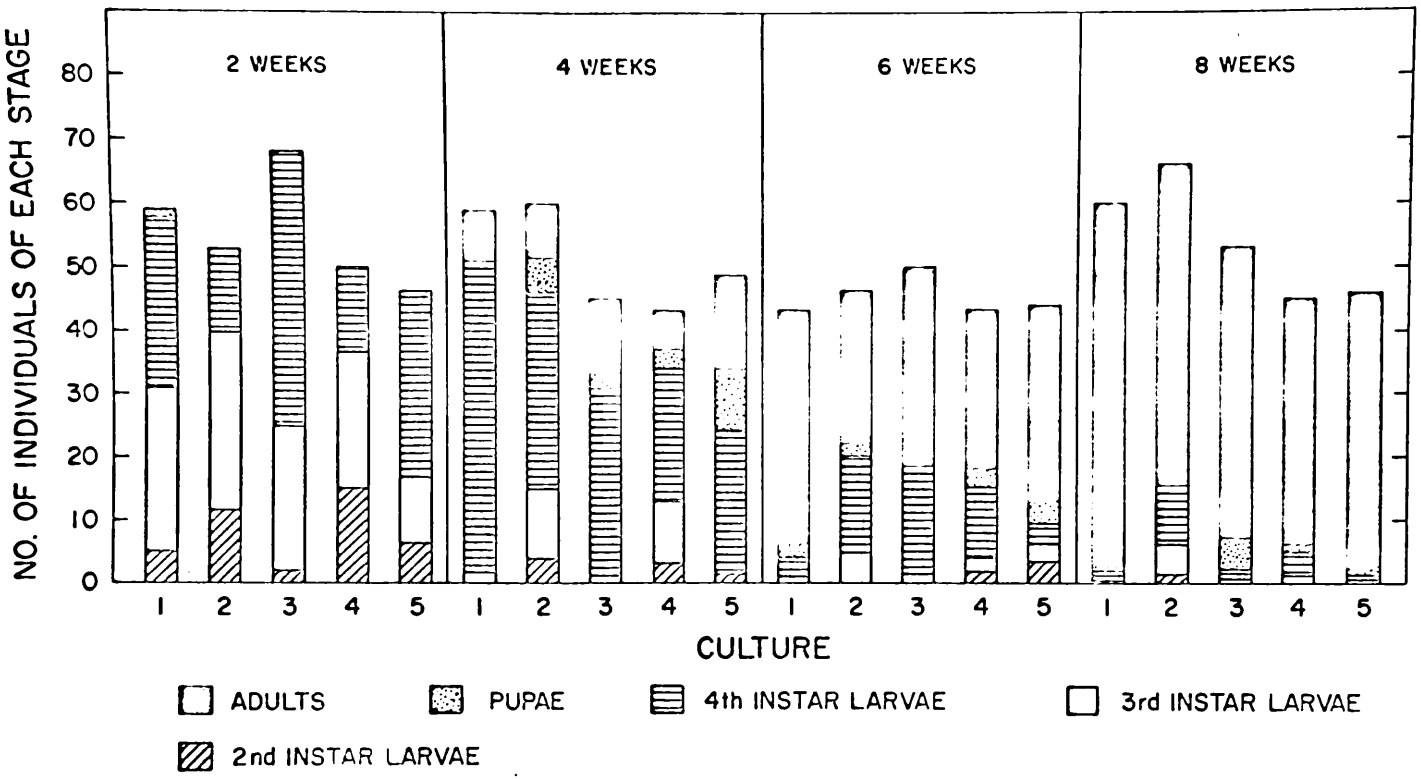

Figure 5. Survival rates of developmental stages present of C. guttipennis in each of the best five protozoan and microorganism cultures over eight weeks: (1) stump-hole microorganisms medium #4; (2) blended stump-hole mixture; (3) Colpidium sp. medium #4; (4) blended leaf-matter mixture; (5) stump-hole microorganisms medium #12.

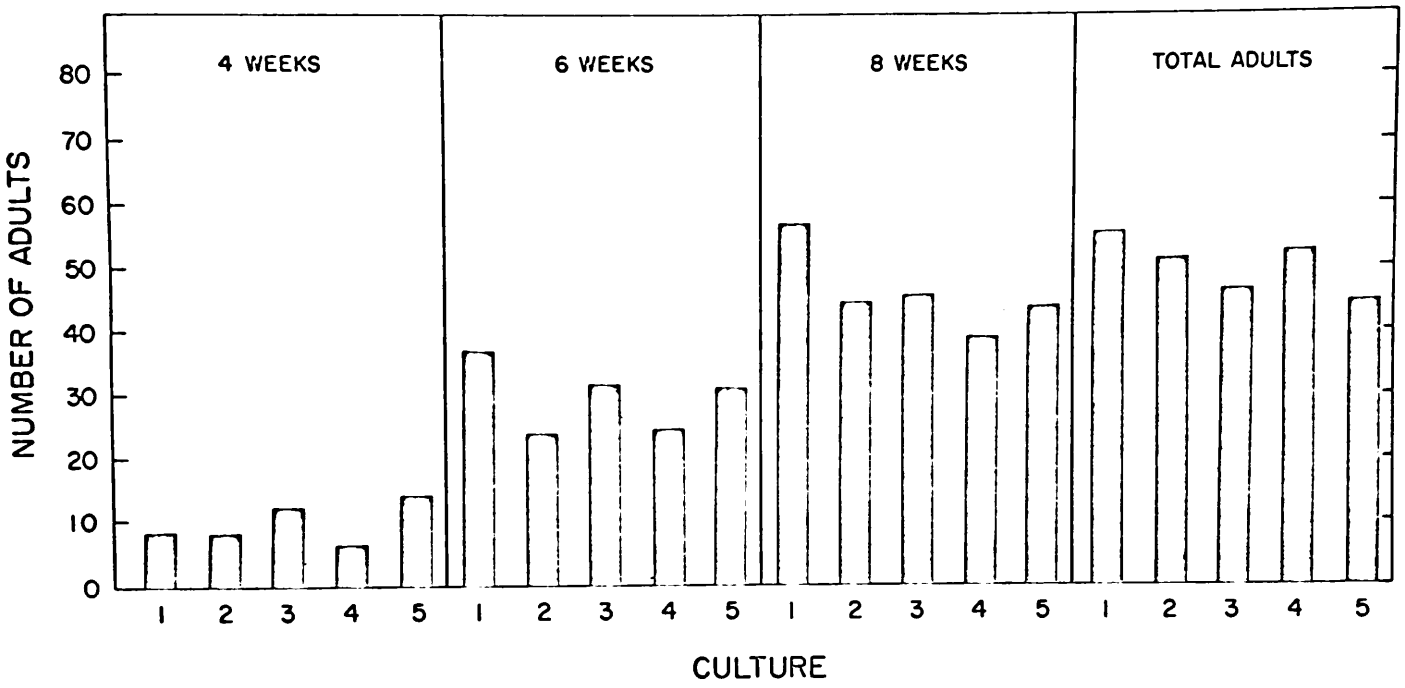

Figure 6. Number of adult C. guttipennis emerged from each of the best five protozoan and microorganism cultures: (1) stump-hole microorganisms medium #4; (2) blended stump-hole mixture; (3) Colpidium sp. medium #4; (4) blended leaf-matter mixture; (5) stump-hole microorganisms medium #12.

Table XII. C. guttipennis adult emergence data for each of the best five protozoan and microorganism cultures for eight weeks.

Culture	Week 2	4	6	8	T.j.
Stump-hole microorganisms medium #4	0	4	9	13	
	0	1	11	16	102
	0	2	8	15	
	0/0	1/8	9/37	13/57	
Blended stump-hole mixture	0	2	7	10	
	0	0	6	11	77
	0	5	6	12	
	0/0	1/8	5/24	12/45	
Colpidium sp. medium #4	0	2	8	10	
	0	3	9	12	89
	0	3	8	12	
	0/0	4/12	6/31	12/46	
Blended leaf-matter mixture	0	2	7	8	
	0	1	6	8	70
	0	2	7	13	
	0/0	1/6	5/25	10/39	
Stump-hole microorganisms medium #12	0	1	9	9	
	0	2	8	14	88
	0	7	6	13	
	0/0	4/14	8/31	7/43	
$T_{i..}$	0	48	148	230	$T... = 426$
$\sum_{k=1}^{4} \sum_{j=1}^{5} X_{ijk}^2$	0	170	1,142	2,252	$\sum_{i}^{3} \sum_{j}^{5} \sum_{k}^{4} X_{ijk}^2 = 3,564$

Table XIII. ANOVA for C. guttipennis adult emergence for each of the best five protozoan and microorganism cultures for eight weeks.

Source	df	SS	MS
Week (W)	2	831.4	415.7**
linear	1	828.1	828.1**
quadratic	1	2.7	2.7
Culture (C)	4	50.8	12.7**
W x C interaction	8	33.2	4.2
W_{linear} x C	4	28.90	7.2*
$W_{quadratic}$ x C	4	4.5	1.1
Error	45	124.6	2.8
Totals	59	1,040.6	

**Significant at the 1% level.

*Significant at the 5% level.

significant. The adults emerging from each of the five cultures had not yet started reaching total adult emergence. This analysis also showed evidence that there was a significant difference at the 1% level between adult emergence in the five cultures. A difference at the 5% level of significance of linear progression was found between each culture.

A Duncan Multiple Range Test was made on the mean adult emergence data of the combined 12 vials from each culture for the three two-week periods. Cultures were ranked from those yielding the greatest number of adults to those yielding the least number of adults. The cultures in that order include: (1) stump-hole microorganisms in medium #4; (2) Colpidium sp. in medium #4; (3) stump-hole microorganisms in medium #12; (4) the blended stump-hole mixture; and (5) the blended leaf-matter mixture. It was concluded that stump-hole microorganisms in medium #4 was greater at the 5% level of significance in the number of adults emerging than the blended stump-hole mixture and the blended leaf-matter mixture. However, it was not significantly greater than Colpidium sp. in medium #4 or stump-hole microorganisms in medium #12. It was also determined that emergence from the culture of Colpidium sp. in medium #4 was not significantly greater than the emergence from the bottom three cultures nor was the emergence from the culture of stump-hole microorganisms in medium #12 significantly greater than the bottom two cultures.

Analysis of the best five cultures was made on data collected from the four vials remaining of each culture for determining total adult

emergence. The data in Table XIV show the total adult emergence for each culture. More adults emerged from the culture of stump-hole microorganisms in medium #4 than from the other four cultures. The adults also emerged in less time than from the other four cultures (see Figure 7). The mean 50% adult emergence time was determined to compare each culture with the adult emergence data collected for the established C. guttipennis laboratory colony using the rearing medium of decaying leaf-matter.

Stump-hole microorganisms in medium #4 provided the most suitable rearing medium for C. guttipennis larvae. There was a higher survival rate, a higher percentage of adult emergence, and the adults emerged over a relatively shorter time period.

2. Comparison between fresh and laboratory-adapted stump-hole microorganisms in medium #4 in rearing C. guttipennis larvae.

The data in Table XV show that a higher survival rate occurred in the fresh stump-hole microorganism culture in each of the two-week periods, in total adult emergence, and in the total survival rate than with the laboratory-adapted culture.

A graphical analysis was made of these two cultures. Figure 8 is a comparison of both the fresh and laboratory-adapted stump-hole microorganisms in medium #4. These data show the total number of surviving individuals of each developmental stage of C. guttipennis at each set of vials. Figure 8A shows that the total number of individuals of each two-week period in each culture differed slightly. Figure 8B shows that considerably more adults emerged from the fresh

Table XIV. Total C. guttipennis adult emergence data for each of the best five protozoan and microorganism cultures.

Protozoan or Microorganism Culture	Total Adults (ΣX)	Total Percent Emergence	Mean Number of Adults per Vial ($\bar{X} \pm SD$)	Mean Days Expired Prior to First Emergence ($\bar{X} \pm SD$)	Mean Total Emergence Time Days ($\bar{X} \pm SD$)	Mean 50% Emergence Time Days ($\bar{X} \pm SD$)
Stump-hole microorganisms medium #4	56	70.0	14.0 ± 1.4	18.0 ± 1.4	28.8 ± 4.0	14.8 ± 2.6
Blended stump-hole mixture	52	65.0	13.0 ± 0.8	27.8 ± 2.2	37.8 ± 3.9	14.5 ± 5.3
Colpidium sp. medium #4	48	60.0	12.0 ± 4.7	30.5 ± 4.0	28.0 ± 4.1	10.3 ± 3.9
Blended leaf-matter mixture	53	66.3	13.3 ± 2.1	36.8 ± 1.5	34.3 ± 1.9	15.8 ± 1.7
Stump-hole microorganisms medium #12	46	57.5	11.5 ± 0.6	20.0 ± 1.8	29.5 ± 5.6	9.0 ± 2.7

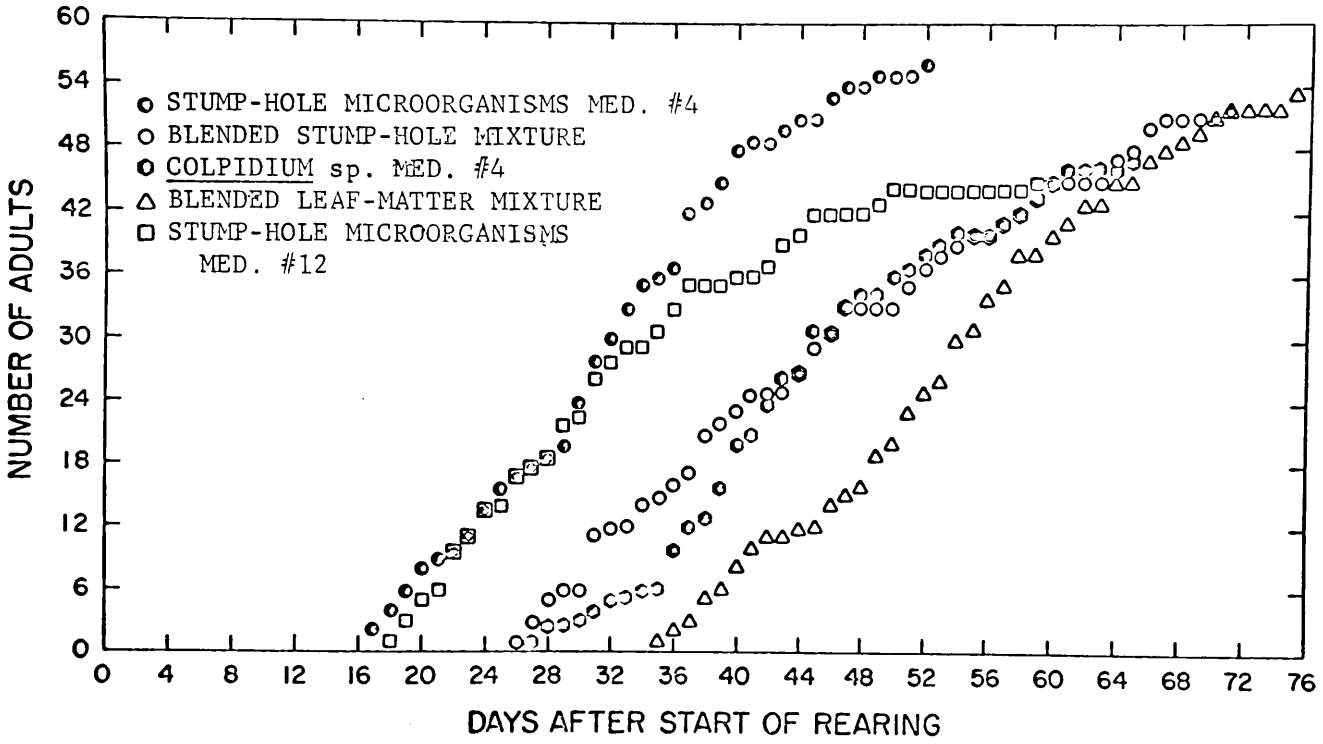

Figure 7. Daily C. guttipennis adult emergence from each of the best five protozoan and microorganism cultures.

Table XV. Mean survival rates of C. guttipennis in the cultures of fresh and laboratory-adapted stump-hole mocroorganisms in medium #4.

Microorganism Culture	2 Weeks ($\bar{X} \pm SD$)	4 Weeks ($\bar{X} \pm SD$)	6 Weeks ($\bar{X} \pm SD$)	8 Weeks ($\bar{X} \pm SD$)	Adults ($\bar{X} \pm SD$)	Total ($\bar{X} \pm SD$)
Fresh stump-hole microorganisms	14.8 ± 4.0	14.8 ± 2.1	10.8 ± 2.5	15.0 ± 1.8	14.0 ± 1.4	13.9 ± 2.8
Laboratory-adapted stump-hole microorganisms	13.0 ± 1.4	11.0 ± 0.8	9.0 ± 2.0	12.3 ± 3.0	10.5 ± 2.5	11.2 ± 2.4

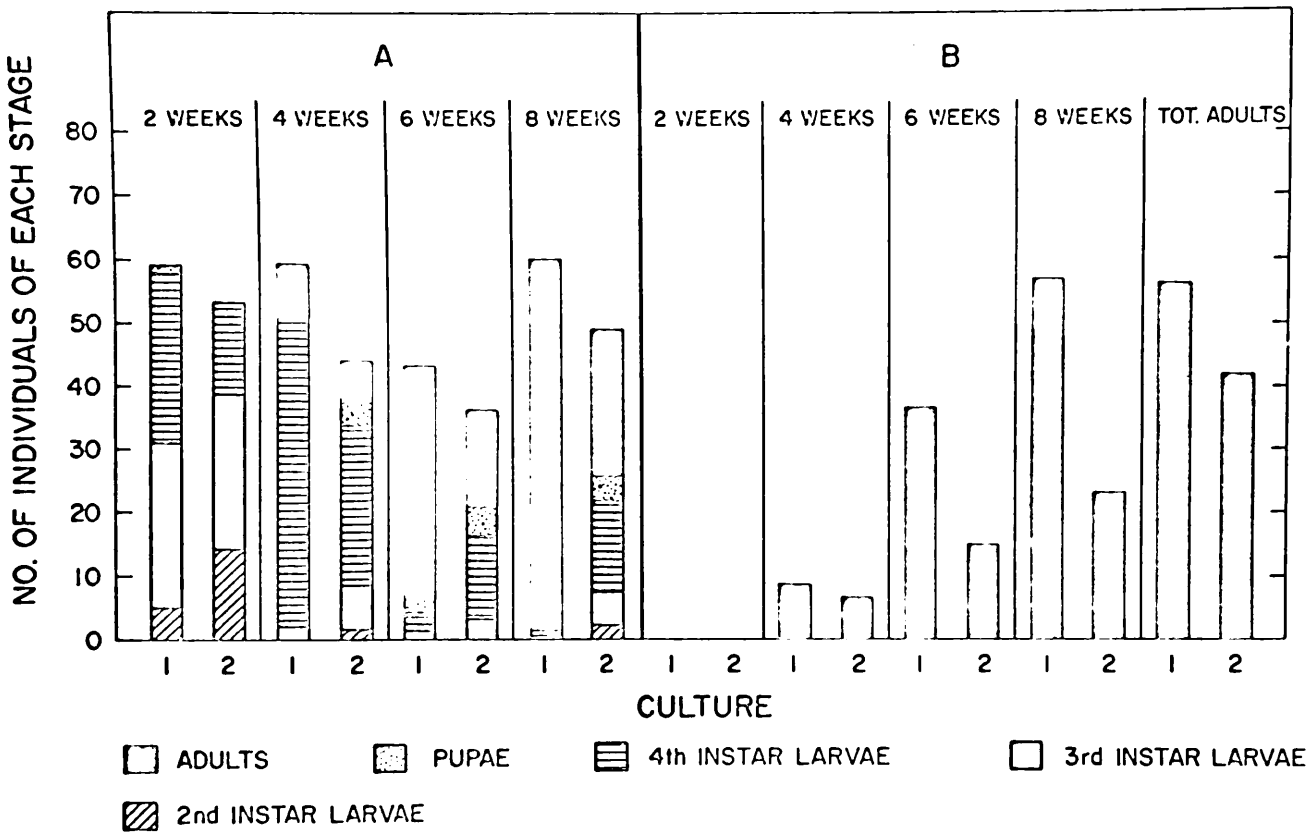

Figure 8. Survival rates of developmental stages present of C. guttipennis over eight weeks from (1) fresh stump-hole microorganisms medium #4 and (2) laboratory-adapted stump-hole microorganisms medium #4. A. number of all developmental stages present; B. number of emerged adults.

stump-hole microorganism culture than from the laboratory-adapted stump-hole microorganism culture.

Statistical analysis shows that the fresh stump-hole microorganism culture is superior to the laboratory-adapted stump-hole microorganism culture in producing more adults at a higher survival rate over a shorter period of time. The data for this analysis are given in Table XVI. The analysis of variance in Table XVII indicates that there is a significant difference at the 1% level in the number of adults emerging from the fresh stump-hole microorganism culture over the adults emerging from the laboratory-adapted stump-hole microorganism culture. This analysis also infers that the linear progression between each of the two media over eight weeks differs at the 1% level of significance. The linear progression of the fresh stump-hole microorganism culture was much steeper over eight weeks. More adults were produced at a faster rate than with the laboratory-adapted stump-hole microorganism culture.

The data in Table XVIII show that the total adult emergence was greater in the fresh stump-hole microorganism culture. The adults emerged earlier and over a shorter period of time than did the adults from the laboratory-adapted stump-hole microorganism culture. Figure 9 graphically presents a comparison of adult emergence.

The mean concentrations of each set of four vials of the dominant forms of Protozoa present in the laboratory-adapted microorganism culture are presented in Table XIX. The initial Protozoa present were those that had been cultured in the medium for four weeks prior to

Table XVI. C. guttipennis adult emergence data for the cultures of fresh and laboratory-adapted stump-hole microorganisms in medium #4.

Culture	Week 2	4	6	8	$T_{\cdot j \cdot}$
Fresh stump-hole micro-organisms	0	4	9	13	
	0	1	11	16	102
	0	2	8	15	
	0/0	1/8	9/37	13/57	
Laboratory stump-hole micro-organisms	0	2	3	5	
	0	2	5	8	44
	0	0	4	8	
	0/0	2/6	3/15	2/23	
$T_{i\cdot\cdot}$	0	14	52	80	$T_{\cdot\cdot\cdot} = 146$
$\sum_{k=1}^{4} \sum_{j=1}^{2} X_{ijk}^2$	0	34	406	976	$\sum_{i}^{3} \sum_{j}^{2} \sum_{k}^{4} X_{ijk}^2 = 1{,}416$

Table XVII. ANOVA for C. guttipennis adult emergence for the cultures of fresh and laboratory-adapted stump-hole microorganisms in medium #4.

Source	df	SS	MS
Week (W)	2	274.3	137.2**
linear	1	272.3	272.3**
quadratic	1	2.1	2.1
Culture (C)	1	138.8	139.8**
W x C interaction	2	65.7	32.9**
W_{linear} x C	1	64.0	64.0**
$W_{quadratic}$ x C	1	1.3	1.3
Error	18	48.0	2.7
Totals	23	527.8	

**Significant at the 1% level.

Table XVIII. Total C. guttipennis adult emergence data for the cultures of fresh and laboratory-adapted stump-hole microorganisms in medium #4.

Protozoan Culture	Total Adults (ΣX)	Total Percent Emergence	Mean Number of Adults per Vial ($\bar{X} \pm SD$)	Mean Days Expired Prior to First Emergence ($\bar{X} \pm SD$)	Mean Total Emergence Time Days ($\bar{X} \pm SD$)	Mean 50% Emergence Time Days ($\bar{X} \pm SD$)
Fresh stump-hole micro-organisms	56	70.0	14.0 ± 1.4	18.0 ± 1.4	28.8 ± 4.0	14.8 ± 2.6
Laboratory-adapted stump-hole microorganisms	42	52.5	10.5 ± 2.5	37.3 ± 13.1	38.8 ± 9.8	19.3 ± 8.4

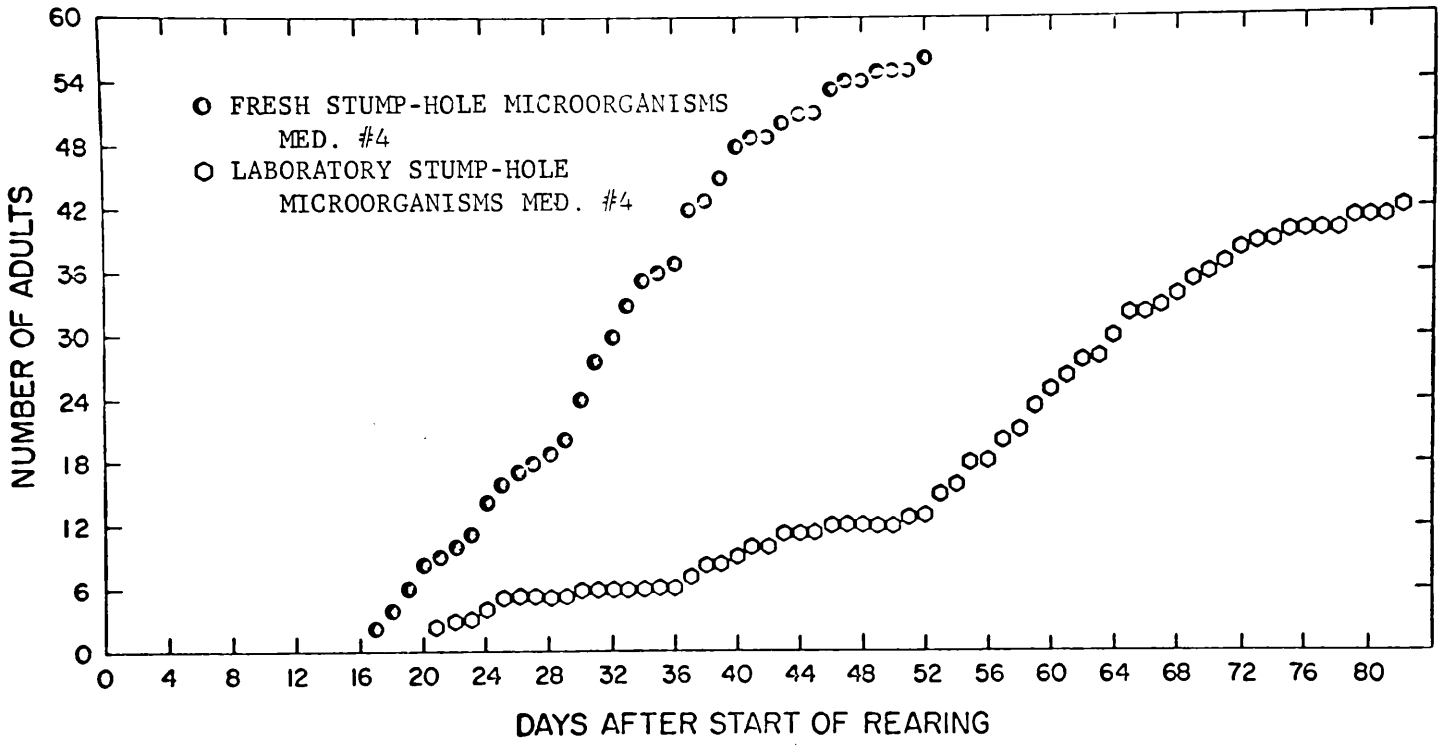

Figure 9. Daily C. guttipennis adult emergence from the cultures of fresh and laboratory-adapted stump-hole microorganisms in medium #4.

Table XIX. Mean protozoan concentrations for eight weeks of laboratory-adapted Protozoa in medium #4 used for rearing C. gutti-pennis larvae.

Week	Mean Protozoan Concentrations (Mean number of individual organisms per ml.)
Initial	Colpoda sp. (400); Loxodes sp. (100); Vorticella sp. (300); Flagellates - Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (100,000)
2 weeks	Colpoda sp. (200); Loxodes sp. (1,000); Vorticella sp. (300); Flagellates - Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (100,000); Rotifers - Philodina sp. (100)
4 weeks	Colpoda sp. (150); Loxodes sp. (600); Flagellates - Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (200,000); Rotifers - Philodina sp. (150)
6 weeks	Colpoda sp. (150); Loxodes sp. (300); Flagellates - Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (150,000); Rotifers - Philodina sp. (200)
8 weeks	Colpoda sp. (200); Loxodes sp. (300); Flagellates - Collodictyon sp., Cyathomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (50,000); Rotifers - Philodina sp. (250)

being used for larval rearing vials.

3. Incorporation of an artificial substrate in larval medium #4.

The comparison was made of the results of C. guttipennis reared in the cultures of fresh stump-hole microorganisms in medium #4, both with and without vermiculite as a substrate. Data similar to the two preceding experiments were recorded.

The data in Table XX show the mean survival rates for the two cultures. The stump-hole microorganism culture with vermiculite resulted in a greater survival rate for each set of vials after two weeks and a greater total survival rate than the same culture without vermiculite added.

A graphical analysis of these two cultures is presented in Figure 10. The total number of surviving individuals of each developmental stage of C. guttipennis at each two-week period, and the total number of adults emerged with each set of vials are compared. Figure 10A shows only a slight difference in the survival rates of the two cultures. A noticeable difference in the developmental rates of the two cultures was observed. In the stump-hole microorganism culture without vermiculite, the developmental rate from early instar larvae to adult production proceeded at a faster rate. However, the data in Figure 10B show that after eight weeks the number of adults emerging from the culture with vermiculite exceeded the number of adults emerging from the culture without vermiculite. Adult production was initially slower when vermiculite was used but ultimately a higher

Table XX. Mean survival rates of C. guttipennis in the cultures of stump-hole microorganisms in medium #4 with and without vermiculite.

Culture	2 Weeks ($\bar{X} \pm SD$)	4 Weeks ($\bar{X} \pm SD$)	6 Weeks ($\bar{X} \pm SD$)	8 Weeks ($\bar{X} \pm SD$)	Adults ($\bar{X} \pm SD$)	Total ($\bar{X} \pm SD$)
Stump-hole microorganisms	14.8 ± 4.0	14.8 ± 2.1	10.8 ± 2.5	15.0 ± 1.0	14.0 ± 1.4	13.9 ± 2.8
Stump-hole microorganisms with vermiculite	14.8 ± 2.5	16.0 ± 1.8	13.8 ± 1.5	17.3 ± 1.7	18.3 ± 1.3	16.0 ± 2.3

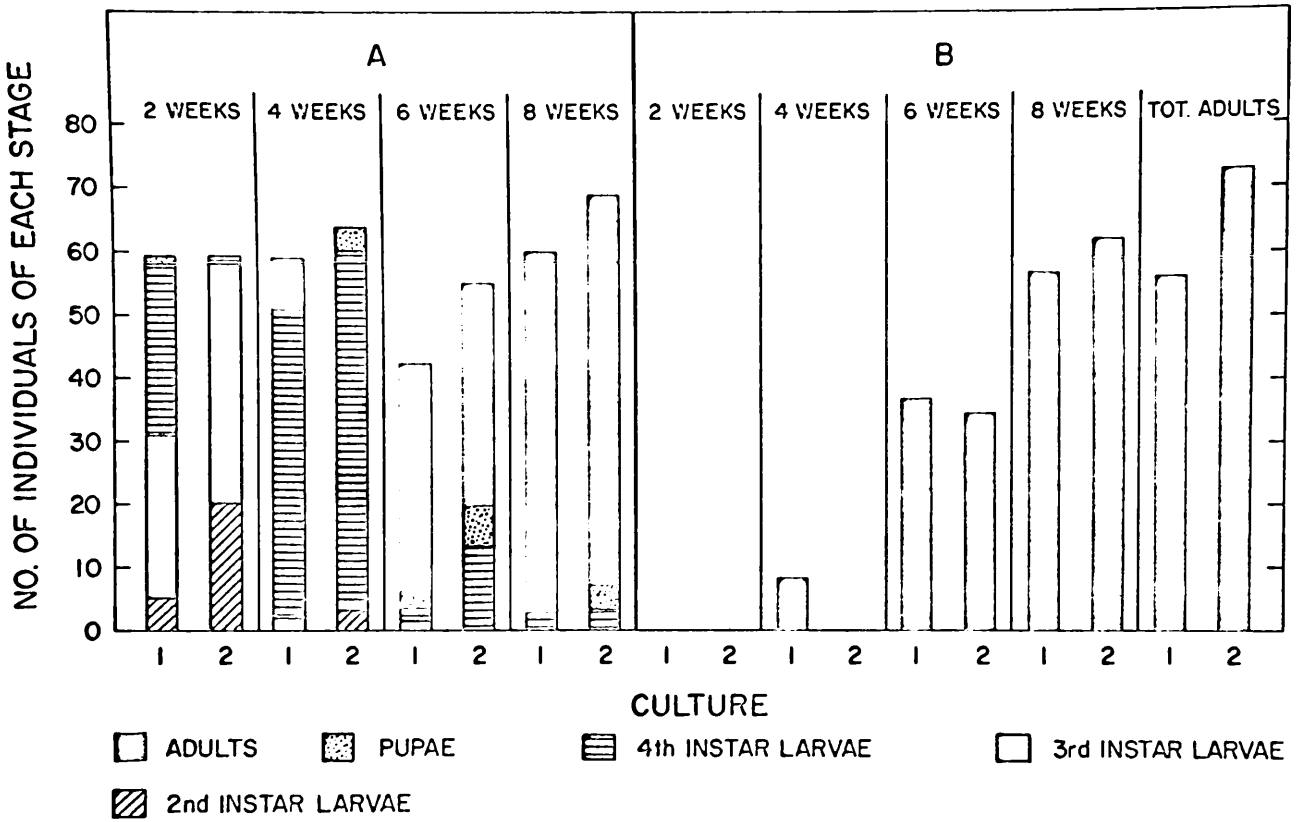

Figure 10. Survival rates of developmental stages present of C. guttipennis over eight weeks from (1) stump-hole microorganisms medium #4 and (2) stump-hole microorganisms medium #4 with vermiculite: A. number of all developmental stages present; B. number of emerged adults.

percent emergence occurred.

The data in Table XXI show the statistical analysis of the adult emergence data from these two media. Table XXII indicates there was no significant difference in the total number of adults emerging from either culture over the eight weeks. However, there was a difference at the 5% level of significance between the linear progression over the eight weeks of each culture. There was a difference in adult production of both cultures at the 1% level of significance over the three two-week periods of adult emergence. This difference was at the 1% level in the linear progression and at the 5% level of significance in the quadratic progression.

A comparison was made of total adult emergence from each culture. The data in Table XXIII show that there was a difference in the percentage of total adult emergence in the two cultures. The stump-hole microorganism culture with vermiculite had a higher percentage adult emergence. The mean time taken for the first adult to emerge was longer, but the mean total emergence time after adults started to emerge was shorter than when vermiculite was not used (see Figure 11).

The mean protozoan concentrations of each set of four vials of the dominant forms of Protozoa present in the stump-hole microorganism culture with vermiculite are presented in Table XXIV. The initial Protozoa present were those in the medium prior to being used for larval rearing vials.

Table XXI. C. guttipennis adult emergence data for the cultures of stump-hole microorganisms in medium #4 with and without vermiculite.

Culture	Week 2	4	6	8	$T_{.j.}$
Stump-hole microorganisms	0	4	9	13	
	0	1	11	16	102
	0	2	8	15	
	0/0	1/8	9/37	13/57	
Stump-hole microorganisms with vermiculite	0	0	10	15	
	0	0	9	14	
	0	0	8	16	
	0/0	0/0	8/35	17/62	
$T_{i..}$	0	8	72	119	$T... = 199$
$\sum_{k=1}^{4} \sum_{j=1}^{2} X_{ijk}^2$	0	22	656	1,785	$\sum_{i}^{3} \sum_{j}^{2} \sum_{k}^{4} X_{ijk}^2 = 2,463$

Table XXII. ANOVA for C. guttipennis adult emergence for the cultures of stump-hole microorganisms in medium #4 with and without vermiculite

Source	df	SS	MS
Week (W)	2	775.1	387.6**
linear	1	770.1	770.1**
quadratic	1	7.5	7.5*
Culture (C)	1	1.0	1.0
W x C interaction	2	11.6	5.8*
W_{linear} x C	1	10.6	10.6*
$W_{quadratic}$ x C	1	0.8	0.8*
Error	18	25.3	1.4
Totals	23	813.0	

**Significant at the 1% level.

*Significant at the 5% level.

Table XXIII. Total C. guttipennis adult emergence data for the cultures of stump-hole micro-organisms in medium #4 with and without vermiculite.

Protozoan Culture	Total Adults (ΣX)	Total Percent Emergence	Mean Number of Adults per Vial ($X \pm SD$)	Mean Days Expired Prior to First Emergence ($\overline{X} \pm SD$)	Mean Total Emergence Time Days ($\overline{X} \pm SD$)	Mean 50% Emergence Time Days ($\overline{X} \pm SD$)
Stump-hole micro-organisms	56	70.0	14.0 ± 1.4	18.0 ± 1.4	28.8 ± 4.0	14.8 ± 2.6
Stump-hole micro-organisms with vermiculite	73	91.3	18.3 ± 1.3	30.3 ± 5.1	22.5 ± 7.1	10.8 ± 7.4

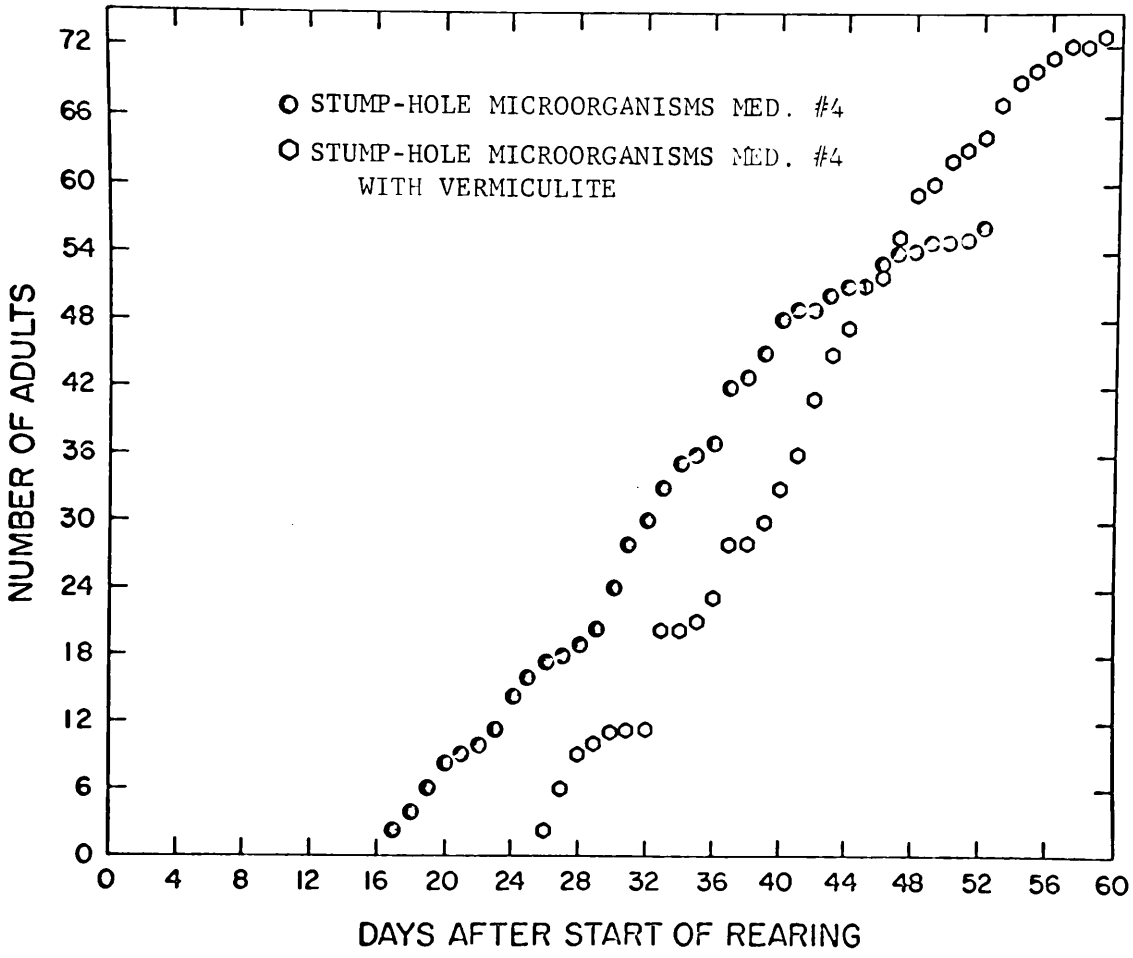

Figure 11. Daily C. guttipennis adult emergence from the cultures of stump-hole microorganisms in medium #4 with and without vermiculite.

Table XXIV. Mean protozoan concentrations for eight weeks of stumphole Protozoa in medium #4 with vermiculite used for rearing C. guttipennis larvae.

Week	Mean Protozoan Concentrations (Mean number of individual organisms per ml)
Initial	Colpoda sp. (1,000); Microthorax sp. (100); Tetrahymena sp. (200); Astasia sp. and Rhabdomonas sp. (200); Flagellates - Cercomonas sp., Collodictyon sp. (100,000)
2 weeks	Cyclidium sp. (1,000); Microthorax sp. (400); Tetrahymena sp. (100); Vorticella sp. (200); Astasia sp. and Rhabdomonas sp. (7,500); Flagellates - Cercomonas sp., Collodictyon sp., Monas sp., Ochronomas sp., Pleuromonas sp., (30,000); Rotifers - Philodina sp. (100)
4 weeks	Cyclidium sp. (2,000); Loxodes sp. (200); Microthorax sp. (3,000); Vorticella sp. (100); Astasia sp. and Rhabdomonas sp. (10,000); Flagellates - Cercomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (30,000); Rotifers - Philodina sp. (150)
6 weeks	Cyclidium sp. (1,000); Loxodes sp. (100); Microthorax sp. (3,000); Vorticella sp. (150); Astasia sp. and Rhabdomonas sp. (20,000); Flagellates - Cercomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (30,000); Rotifers - Philodina sp. (100)
8 weeks	Microthorax sp. (7,500); Astasia sp. and Rhabdomonas sp. (5,000); Flagellates - Cercomonas sp., Monas sp., Ochromonas sp., Pleuromonas sp. (7,500); Rotifers - Philodina sp. (150)

F. Revised Rearing Techniques for C. guttipennis.

The development of an artificial larval medium allowed for revisions in the rearing technique for the laboratory colony of C. guttipennis. Changes were made in the larval rearing techniques and in the handling of egg laying vials, but no changes were made in adult maintenance.

The two-dram vials used for egg laying originally contained water from stored decaying leaf-matter. This was replaced with a one-week-old culture of the stump-hole microorganisms in medium #4. Seven-hundred ml of the medium were made up each week for the egg vials. The medium was inoculated with 20 ml of filtered stump-hole water. After one week of infusion, the culture was added to egg vials. One egg vial was used for each adult holding cage. Filter paper was rolled to fit the inner surface of the vial and the vial half-filled with the one-week old culture. The culture was used for filling egg vials for one week and then discarded. Egg vials were removed each day from the adult holding cages. They were placed into an egg incubator, as described by Hair and Turner (1966), for four days to allow for egg hatch before being transferred to larval rearing containers.

For the larval rearing containers, 1,400 ml portions of medium #4 were made up. After allowing the medium to set overnight, each portion was combined with 700 ml of vermiculite previously soaked and cleaned in distilled water. The vermiculite was pre-soaked so it would readily sink to the bottom of the larval medium. It was then placed into the black-painted glass jars used for larval rearing containers

(Figure 2). The medium was then inoculated with 40 ml of filtered stump-hole water. After one week of infusion, the contents from egg vials were added to these containers. Each egg vial contained first instars of C. guttipennis. Two new rearing containers were started each week. Each rearing container was held for no longer than two months.

Larval medium kept in the laboratory for filling egg vials was also used for maintaining the levels of medium in each rearing container. Additional medium was added to each rearing container once a week to replace the water which had evaporated. The addition of more medium insured the maintenance of the population of the microorganisms.

V. DISCUSSION AND CONCLUSIONS

The adult emergence data from the laboratory colony of C. guttipennis showed a decreasing trend in adult production over the two-year study period. Several causes could have been responsible for this trend. The established colony could be losing genetic vigor after more than eight years of colonization. This reduced vigor could result in reduced mating and possibly unequal sexual ratios of emerging adults. In this study, the larval rearing medium was investigated as a possible reason for the decreasing adult production. The suitability of a larval rearing medium may have significant affects upon adult production. The original larval medium was of an array of living organisms in organic leaf-matter. The contents of this medium were in a constant state of change. Whenever new larval rearing containers were made with stored decaying leaf-matter, the ingredients varied in their organic makeup and in the number and kinds of living organisms.

In addition to the fluctuating ingredients, a moth fly (Psychodidae, Telmatoscopus sp.) occasionally became established as a detrimental contaminant in the stored leaf-matter in the laboratory and was found in great numbers in the larval rearing containers. Jones (1964, 1966) reported psychodids contaminated the rearing medium used for his colony of C. variipennis. These flies were detrimental to the colonized Culicoides larvae. Jones (1959), studying the breeding sites of C. variipennis sonorensis in buildings of human-sewage disposals, found a small number of psychodids always present in

association with the Culicoides larvae. A dense population of one apparently precluded a large population of the other. These flies frequently laid eggs in the egg laying vials of the C. guttipennis colony. The psychodids were eliminated immediately from the laboratory with the development of an artificial larval medium made fresh each week.

A comparison between the sterile diet and the non-sterile decaying leaf-matter showed that the larvae were dependent on living organisms for their nutritional needs. These living organisms included several species of Protozoa, bacteria, algae, fungi, and rotifers. The larvae apparently feed on a combination of any or all of these organisms which are apparently of direct nutritional value, or at least the larvae are dependent upon some accessary factors furnished by them.

As indicated by other workers, the majority of ceratopogonid larvae appear to rely on various microorganisms for all or part of their nutritional needs. Most larvae that appear to be unselective in their feeding habits perhaps merely browse through organic matter in search of microorganisms. Baumberger (1919) stated that the use of microorganisms as food is widespread among insects and is a direct response to their high nutritive value. Glaser (1924) commented that the larval stages of flies are dependent upon certain accessary growth factors which are obtained from bacteria, yeast, and other living organisms. Sonleitner (1964) supported these two authors stating that the difference between dead and living food is not always clearcut, because in many cases the insects are actually feeding on

microorganisms or relying on them for accessary factors. Trager (1953) stated that in the absence of microorganisms it becomes evident that insect larvae require many different nutrients, including a long list of accessary growth factors.

Most larval diets include media that are suitable for a variety of bacteria, fungi, algae, and Protozoa. An artificial larval medium was the objective of the present study, and this medium was sought in the form of a microorganism culture using simple ingredients easily prepared in the laboratory. The stump-hole microorganism culture found effective in rearing C. guttipennis was in an artificial medium. The medium ingredients were standardized and the media that were prepared each week were inoculated with stump-hole water from a common source.

A problem encountered in trying to develop an artificial larval medium using various microorganism cultures was that certain contaminant microorganisms were present in nearly all of the cultures used in the experiment. These contaminant microorganisms prevented more accurate evaluations of single species of Protozoa. Many of these contaminant microorganisms were already present in the single species cultures when first obtained from Carolina Biological Supply Company. Apparently they were not considered detrimental to the normal culture of these species. Other contaminant organisms found among the cultures probably came from the air, from the use of non-aseptic techniques in the laboratory, and from the use of non-sterilized eggs. It was also observed that even the blank media used for evaluating medium nutrients in rearing larvae contained contaminant microorganisms.

Small numbers of adults were produced in some of the blank media as a result of this.

That Protozoa are readily found in the air is verified from several studies. Puschkarew (1913) estimated that there were about 3.5 viable protozoan cysts per cubic meter of air. Puschkarew obtained many small amoebae, flagellates, and a ciliate (Colpoda sp.). More recently Gregory (1960) and Schlichting (1961) showed several viable Protozoa present in the air.

There was constant fluctuation in the population levels in protozoan and microorganism cultures. Different microorganisms have different growth rates. Jahn (1934) listed some of the factors affecting growth rates including: (1) changes in quantity and quality of available food material, both bacteria for holozoic and dissolved substances for saprozoic forms; (2) changes in the concentration of unidentified waste products of metabolism; (3) changes in pH, both directly and indirectly as such changes affect the dissociation of components of the medium or the ecological sequence of the bacteria; (4) changes in oxygen tension; (5) changes in carbon dioxide tension; (6) changes in temperature; (7) changes in light; and (8) fluctuations and changes in numerous specific chemical compounds which are produced by bacterial decomposition.

Several explanations can be speculated as to why the speed of larval growth and survival rates of C. guttipennis differed among the cultures used in this study. The microorganisms surely had major affects on the larvae. The factors affecting the population structure of

microorganisms probably also affected the growth and survival rate of insect larvae breeding in the same medium. Microorganisms that became numerous in some cultures may have added toxic material in the medium. Microorganisms present could have competed with the larvae for food and thus affected larval growth and survival. Also, they could have competed with other microorganisms that were nutritionally important to the growing larvae. That microorganisms furnish insects with nutritional needs has already been discussed. The success of some cultures, therefore, relied on adequate numbers of proper microorganisms for meeting some of the nutritional needs of the larvae. Some cultures probably did not have a suitable community structure of microorganisms for development of the larvae.

The medium itself could have been a factor in the success or failure of the cultures. Some media may have had ingredients producing toxic affects directly on the larvae and could have been detrimental to them. Other ingredients could have been beneficial for some cultures by providing essential nutrients to the larvae.

The factors contributing to the possible affects on larval growth and survival combine to make the larval medium a highly complex entity. Interactions that must occur between these factors need to be more fully understood in order to really evaluate affects between different cultures.

The ideal larval medium is one that is able to withstand the pressure of the growing Culicoides larvae, to furnish the larvae with a relative non-toxic environment to grow in, and to provide the

developing larvae their optimal nutritional needs. The culture of stump-hole microorganisms in medium #4 satisfied the nutritional needs of C. guttipennis larvae. This culture was more effective than the established rearing medium of decaying leaves. The data in Table XI showed that culture media inoculated with stump-hole microorganisms were all relatively successful in rearing C. guttipennis. Three of the five cultures in which there was a total survival rate of at least 50% were those using stump-hole microorganisms. In addition, relatively high survival rates of C. guttipennis larvae resulted when those cultures using medium #4, a simple hay infusion, were used. Apparently C. guttipennis larvae receive adequate nutrient material from both sources.

Ideally, an artificial larval medium developed for a laboratory reared insect should not be dependent on material from the natural breeding habitat. The weekly inoculation of the culture media with organisms taken directly from the stored stump-hole contents indicates a definite dependency on the natural breeding habitat of C. guttipennis larvae. When the laboratory-adapted stump-hole microorganism culture was used to rear C. guttipennis larvae, there was a significant decrease in survival rate and adult emergence. Apparently certain necessary microorganisms were not able to become established in a closed laboratory reared culture using the media described in this study.

When vermiculite was used as an artificial substrate in the stump-hole microorganism culture, an even higher survival rate of C. guttipennis larvae resulted. Adult emergence exceeded 90%, compared to 70%

without vermiculite. The vermiculite apparently added something to the culture to have direct effects upon larval development. It is possible that vermiculite was a factor in absorbing toxic waste material of larval and microorganism metabolism. This would have made the environment for the developing larvae cleaner and more desirable. The larvae in the vials containing vermiculite were constantly searching and moving about under the vermiculite. Apparently the microorganisms and nutrients were concentrating among the vermiculite particles. The larvae would, therefore, be able to have more food material available in concentrated areas. The presence of vermiculite could also have affected the population numbers and kinds of microorganisms present. Evidence from the concentration counts of Protozoa in the vermiculite experiment indicated that some protozoans differed in numbers in the medium containing vermiculite from when vermiculite was not used. The success or failure of certain protozoans to fluorish in the vermiculite medium may have been a key factor in its success as a larval medium. Also, the larvae were probably able to protect themselves from possible cannibalism by hiding among the vermiculite particles. All of these factors likely combine to make the stump-hole medium incorporating vermiculite satisfactory for rearing the larvae.

The results with the artificial larval medium of stump-hole microorganisms in medium #4 incorporating vermiculite can be compared to those obtained from the adult emergence data for two years from the established laboratory colony of C. guttipennis. The mean time elapsed between the setting up of a rearing container and the first adult

emergence in the established colony was 23.0 days. The stump-hole microorganism culture time was 30.3 days. The total emergence time using the artificial medium was 22.5 days. This was considerably shorter than the total emergence time of 77.4 days in decaying leaf-matter. The time for 50% of the adults to emerge also was much shorter using the artificial medium (10.8 days) as compared to decaying leaf-matter (25.9 days). Adults were slower to emerge initially from the artificial stump-hole microorganism culture; but when emergence started, more adults emerged over a much shorter period of time.

It seems evident that the artificial larval medium of stump-hole microorganisms in medium #4 incorporating vermiculite was a better larval medium and should be successful in incorporating into the rearing procedure of the established laboratory colony.

The efficiency in adult production can be estimated by adding the value of adult emergence to the percentage of egg viability. Thus, with an egg viability of 67% and a mean total adult emergence from viable eggs of up to 91% from the stump-hole microorganism culture incorporating vermiculite, 58% of eggs laid by C. guttipennis females can potentially produce adults.

The use of an artificial larval medium incorporating stump-hole microorganisms to rear C. guttipennis shows a possible enhancement of this insect to its natural habitat. This enhancement is especially significant since the C. guttipennis larvae used for the present study were from an established colony which had been maintained in the laboratory for over eight years, without introducing wild flies from

the field.

Decaying leaf-matter has a community structure of microorganisms that is somewhat different from that found in a tree- or stump-hole habitat. The microorganisms making up the community structure in this tree- and stump-hole environment may therefore be partially responsible for this insect having selected this particular type of habitat. Further studies dealing with the tree- and stump-hole community populations should help understand why C. guttipennis selects this habitat for breeding. Attention should be directed to the food-chain between the larvae and microorganisms.

Further studies should also be made with the newly developed artificial larval medium. The optimum temperature and pH range should be determined for larval growth and development that is also satisfactory to insure the maintenance of large populations of microorganisms in the medium. Studies to determine the maximum density levels possible for developing larvae would also be desirable. Other studies would be to determine what the larvae actually feed on. From the present study it was observed that certain microorganisms may be more desirable as larval food than others. Also, the use of the contents from different tree- and stump-holes in inoculating the larval medium could result in differences in the survival and developmental rates of C. guttipennis larvae.

Since the colony has been adapted to the laboratory for over eight years, wild strains of C. guttipennis would most certainly have some effects on the vigor of the colony. Studies would also be desirable

on various aspects of mating and in the determination of the length and number of gonadotropic cycles in individual females. Studies of this nature would help better understand the efficiency and desirability of the reproductive potential of this insect in maintaining a mass population of insects desired for a laboratory colony.

With the development of an artificial larval medium for colonized C. guttipennis, similar diets could be developed for colonizing other Culicoides spp. A relatively simple microorganism medium, inoculated with microorganisms from several different breeding habitats of Culicoides could be used for rearing several Culicoides spp. in the laboratory.

VI. SUMMARY

There has been an increased need in recent years for studies dealing with the bionomics, nutrition, physiology, disease-vector relationships, and control methods of Culicoides spp. Insufficient laboratory-reared material has hindered the progress of many of these studies. An important factor holding up the development of laboratory culture techniques is the lack of knowledge of the nutritional requirements of Culicoides larvae. This study attempted to develop an artificial larval medium for colonized C. guttipennis and to create a uniform condition for larval growth. This would thus increase the suitability of the colony for further studies.

An evaluation was made of the rearing technique used for an established laboratory colony of C. guttipennis. The rearing medium and technique used could have been partially responsible for a decrease in adult production.

An experiment was conducted to verify that C. guttipennis larvae were carnivorous. Decaying leaf-matter was previously sterilized to kill all living organisms present and compared with non-sterilized leaf-matter in rearing C. guttipennis larvae. The non-sterilized leaf-matter possessed several kinds of protozoans, rotifers, and other microorganisms. Larvae in the sterile leaf-matter did not develop beyond the first instar. Larvae in the non-sterilized leaf-matter were able to complete development. Research was directed toward culturing various microorganisms in artificial media to develop an artificial rearing medium for the C. guttipennis larvae.

Several protozoan media were tested for successfully rearing various kinds of Protozoa and other microorganisms. The protozoan and microorganism cultures with satisfactory concentrations of their appropriate microorganisms were used for rearing C. guttipennis larvae. The culture most successful in producing more adults over a shorter period of time consisted of stump-hole microorganisms in a hay-infusion medium containing fine-mesh hay, flour, and water. This larval medium was also inoculated with fresh stump-hole microorganisms taken directly from stored stump-hole contents.

An experiment was conducted to compare this successful culture with one containing the same medium but with stump-hole microorganisms adapted to an artificial laboratory environment. This experiment was attempted to minimize the dependency of the developing larvae to their natural habitat. However, it was determined that the fresh stump-hole microorganism culture was more effective in rearing larvae and producing adults. Some forms of microorganisms from this habitat, not able to become completely established in an artificial one, are apparently needed for C. guttipennis larval development.

The addition of vermiculite as an artificial substrate for the stump-hole microorganism culture proved effective in rearing C. guttipennis larvae. More adults were produced with this substrate than when the culture was used alone. The vermiculite apparently makes the larval environment more desirable by absorbing waste material in the culture. Also, the microorganisms present in the culture appear to increase in number becoming more available to the larvae as food.

The development of a successful artificial larval medium for C. guttipennis will aid further studies in developing laboratory colonies of related Culicoides spp. in the laboratory. Using a simple medium, such as a hay infusion, Protozoa and microorganisms found from the breeding sites of several Culicoides spp. can be cultured.

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VIII. VITA

Ralph Edward Williams was born in Indianapolis, Indiana, August 13, 1949, to Ernest Quincy and Norma Gertrude (Johnston) Williams. He graduated from Emmerich Manual High School in June, 1967. He entered Purdue University, September, 1967, and received a B.S. Degree in Agriculture, majoring in Entomology, in June, 1971. He has been enrolled in the Graduate School of Virginia Polytechnic Institute and State University since September, 1971, and is pursuing the M.S. Degree in Entomology. He has been an NDEA fellow while at V.P.I. and S.U. He was married June 7, 1970, to Deborah, daughter of Adrian A. and Nellie (Newton) Stroud. He is a member of the American Mosquito Control Association, the Entomological Society of America, and Phi Sigma Honorary Biological Society.

Ralph E. Williams

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AN ARTIFICIAL LARVAL MEDIUM FOR COLONIZED CULICOIDES GUTTIPENNIS (COQUILLETT) (DIPTERA: CERATOPOGONIDAE)

by

Ralph Edward Williams

(ABSTRACT)

An artificial larval medium for colonized Culicoides guttipennis was developed. Microorganisms in decaying leaf-matter were present in the original laboratory diet. These were believed to be the main food of C. guttipennis larvae. This was verified by sterilizing the leaf-matter to kill the organisms present. Larvae did not develop beyond the first instar in this medium, but adults were produced from non-sterilized leaf-matter.

The objective was to develop cultures of various microorganisms and find one that would be best for rearing C. guttipennis larvae in producing more adults. A simple hay-infusion medium inoculated with stump-hole microorganisms was found more effective than all other cultures screened in rearing the larvae. This culture consisted of stump-hole microorganisms inoculated fresh each week from stored stump-hole contents. It was compared with the same culture containing stump-hole microorganisms completely adapted to a laboratory environment. The former was better in rearing C. guttipennis larvae. A final experiment evaluated the incorporation of vermiculite as an artificial substrate. This proved successful in rearing larvae and producing large numbers of adults. This culture was superior over the larval rearing

medium of decaying leaf-matter.

Development of a successful artificial larval medium for colonized C. guttipennis will aid in establishing other laboratory colonies of related Culicoides species.