

# PREPARASITIC STAGES IN THE LIFE HISTORY OF THE CATTLE HOOKWORM (*BUSTOMUM PHLEBOTOMUM*)<sup>1</sup>

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## INTRODUCTION

The occurrence of hookworm infestation in cattle in the United States attracted considerable attention several years ago because of the supposed etiological relation of these parasites (*Bustomum phlebotomum*) to a disease of cattle commonly known as "salt sick." Following the report of Stiles (14),<sup>2</sup> who called attention to the occurrence of hookworms in cattle in Texas, Dawson (5) pointed out the resemblance between the clinical picture of hookworm anemia in man and "salt sick" in cattle, and in 1906 the same writer expressed a more definite opinion regarding the relation of cattle hookworms to "salt sick," which he characterized as "an acute or chronic parasitic disease manifested at first by low fever, diarrhea, loss of appetite; soon becoming chronic, with continuance of low fever, constipation, loss of appetite, progressive emaciation and pronounced anemia, which in many cases terminates fatally." While it has not been conclusively established that the condition in cattle commonly known as "salt sick" is actually due to hookworm infestation, the fact that cattle heavily infested with these parasites develop a progressive anemia, as shown by Dawson (6), has been confirmed by other observers, notably by Reisinger (13).

With a single exception, the published reports dealing with hookworm disease in cattle contain no information based on original investigations concerning the life history of *Bustomum phlebotomum*. This exception is a paper by Conradi and Barnett (4) which contains a very brief account of the development of the egg up to the infective stage, but lacks essential details. Since precise information concerning the preparasitic stages in the life history of strongyle parasites is generally useful in connection with control measures against these parasites, it is important that pertinent

facts concerning the life cycle of cattle hookworms become available in order that such control measures may be devised. Hence the observations and experiments described in this paper were undertaken. As the work progressed some observations and experiments of a less practical nature were included in order to compare the behavior of cattle hookworm larvæ with those of other strongyle parasites.

## MATERIAL AND METHODS OF INVESTIGATION

Specimens of hookworms from cattle, collected in the course of post-mortem examinations of the viscera, were washed several times in physiological salt solutions and then chopped with a pair of fine scissors to liberate the eggs from the uteri of the females. With the aid of a pipette chopped-up worm material was added to the surface of feces and charcoal mixtures in glass dishes and bottles (solid culture media) and to water and filtrate from boiled feces in staining jars and Petri dishes (liquid culture media).

The solid culture media were made up as follows: Sheep or cattle feces were boiled in water and filtered through ordinary filter paper. Sufficient charcoal was added to the filtrate to make a thick paste, which was spread out in Petri dishes, wide-mouth bottles, and other glass containers, care being taken to smooth down the surface of the medium and to keep the latter moist by adding water whenever necessary in order to make up the loss due to evaporation. When diluted with water sufficient to make a transparent medium, the filtrate from the boiled feces was found to be an excellent culture medium for the examination of contents from day to day. A thin layer of the liquid was added to the glass containers, thus affording a supply of oxygen, which is requisite to rapid development. Culture media were kept at room temperature (70°

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<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 458.

to 80° F.) and examinations were made by mounting the glass vessels containing liquid media directly on the stage of the microscope for general observation, and resorting to isolation of larvæ whenever detailed observations on structure or behavior were desirable.

Inasmuch as not all eggs hatch at the same time, because of variation in rate of development and various other factors, newly hatched larvæ were isolated and mounted on cover glasses inverted over hollow ground slides or over ordinary slides to which temporary chambers were sealed. Thus the changes undergone by the larvæ could be followed very readily without such confusion as would result from having larvæ at various stages of development

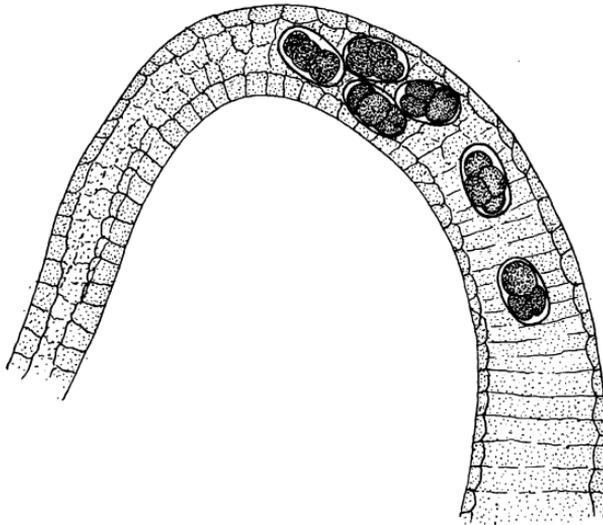


FIG. 1.—Portion of uterus containing segmented eggs

in the same dish. If precautions are taken to secure good-sized drops, and if hanging-drop preparations are properly sealed, the changes in the larvæ from the time of hatching to the infective stage may be followed without adding additional fluid, thus avoiding the need of removing the cover glass at various intervals. In one series of observations eggs and larvæ were secured from day to day from solid culture media and their development was compared with that of eggs and larvæ in liquid media. No noteworthy differences in rapidity of development were found; therefore observation on the rapidity of development in subsequent series of cultures was limited to those made on liquid media.

#### DESCRIPTION OF THE EGG

The egg of *Bustomum phlebotomum*, in common with eggs of other stron-

gyles, is thin-shelled, elliptical in shape, and exhibits considerable range in size (52 to 106  $\mu$  in length, by 43 to 60  $\mu$  in width). In a series of measurements involving more than 100 eggs only 10 per cent of the eggs exceeded a length of 100  $\mu$  and only about 5 per cent were shorter than 75  $\mu$ . The majority of eggs measured were from 85 to 95  $\mu$  long and slightly over 50  $\mu$  wide. No definite correlation was found between length and width of eggs. Some long eggs are comparatively narrow and some present an almost spherical appearance.

#### DEVELOPMENT OF THE EGG

Conradi and Barnett (4) state that before the egg is passed from the body of the host it is in the mulberry stage. These writers also note that partial incubation of the egg in the intestine of the host seems to be important, since eggs that were taken from the host's intestine and thus missed the partial incubation period in the intestine showed a heavier mortality than eggs collected from feces. The present writer noted that eggs begin to segment in the uterus of the female parasite, as shown in Figure 1. Eggs obtained as a result of chopping freshly collected worms are in the one-, two-, and four-cell stages, and rarely in the eight-cell stage. At room temperature (70° to 80°

F.) the development of such eggs takes place rather slowly. After 24 hours of incubation it was noted that comparatively few eggs had reached the mulberry stage; and after 72 hours of incubation comparatively few embryonated eggs, with embryos moving in the shells, were found. Ninety-six-hour cultures usually showed free larvæ as well as embryonated eggs, the latter hatching in the course of a few hours. According to Conradi and Barnett (4), at a temperature of 48° to 60° it required 31 days for the eggs to hatch. These writers also report that eggs obtained from feces on February 26 hatched on February 28; but they do not give the temperature at which the eggs were incubated. According to observations by the present writer, eggs at various stages of development undergo disintegration in culture media not only because of the presence of bacteria in

cultures, but also because of other factors probably present within the egg as well as in the surrounding medium.

#### PREINFECTIVE LARVÆ

The first-stage larva exhibits lively movements, twisting its body in typical nematode fashion. Several first-stage larvæ were kept under observation about seven hours, during which period they were incessantly active, moving about with vigorous flexures of the anterior portion of the body aided by the propelling movements of the long, slender tail.

These larvæ are commonly from 420 to 450  $\mu$  long, or somewhat longer, by 20 to 25  $\mu$  wide. The first third of the body is the broadest, the remaining portion tapering gradually and terminating in a slender tail. Structurally, the organism is relatively simple, its most conspicuous organs being an alimentary canal consisting of an esophagus with a terminal bulb and a straight intestine (this being densely granular in contrast to the less granular esophageal region), and a genital primordium.

About 24 hours after hatching, first-stage larvæ became very sluggish, a physiological condition (lethargus) which precedes molting. Examination of such sluggish larvæ with high magnification generally showed a separation of the cuticle in the cephalic extremity, revealing a newly formed cuticle underneath the old one.

The span of life of the second-stage larvæ is comparatively brief, since 18 hours after the first lethargus was observed the second lethargus was found to be in progress. The second-stage larva shows a slight increase in size (490 by 25  $\mu$ ) and is less coarsely granular than the first-stage larva. Before its quiescent stage preparatory to the final molt it shows moderate activity, less marked, however, than that of the first-stage larva.

Although in a 6-day-old culture several quiescent larvæ undergoing the final molt were found, third-stage larvæ were generally not observed before the eighth day after making the culture. The second lethargus lasts at least 24 hours.

The duration of the preinfective stages in the life of the larvæ may be increased by subjecting them to low temperatures, which retard their development. Thus first-stage larvæ were prevented from further development for a period of 10 days by being kept

in water in a refrigerator at a temperature of 8° C. While a number of larvæ degenerated during that period, others were still alive and showed no changes of structure or other evidence of having molted. Meanwhile larvæ from the same lot at room temperature under-

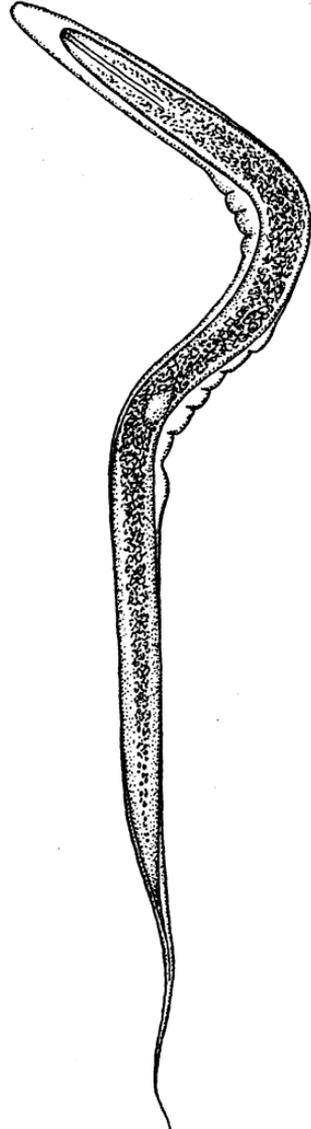


FIG. 2.—Outline drawing of infective larva from solid culture medium showing protective sheath

went their normal course of development.

#### INFECTIVE LARVÆ

In rich 8-day-old cultures third-stage larvæ were found in abundance. These organisms are readily recognized by their greater transparency, which in-

creases as they are kept alive in cultures. The earlier-stage coarser granules practically disappear and the larvæ also retract within the shells, showing quite distinctly the protective sheath, which is usually wrinkled (fig. 2).

The infective larvæ are generally from 500 to 540  $\mu$  long by 20 to 27  $\mu$  wide, although infective larvæ slightly less than 500  $\mu$  long were found on several occasions. The distance from the cephalic extremity to the base of the esophagus was found to vary from 125 to 145  $\mu$ . The diameter of the esophagus at the base is 10  $\mu$ . The nerve ring has a diameter of 5  $\mu$  and is usually situated 60  $\mu$  from the cephalic extremity. In one specimen the distance was found to be 78  $\mu$ , while in another specimen it was only 50  $\mu$ . The distance from the anterior extremity to the genital primordium is slightly in excess of 200  $\mu$ . The genital primordium is 10  $\mu$  long by 5  $\mu$  wide. The tail corresponds roughly to the terminal sixth of the total body length (fig. 4).

Third-stage larvæ obtained from solid cultures have only the protective sheath, the first cuticle having been discarded. Third-stage larvæ obtained from liquid cultures almost invariably show two sheaths, indicating that the first as well as the second sheath has been retained (fig. 3). This observation was verified repeatedly in liquid cultures made from different lots of worms. The first cuticle (first molt) is generally rigid, whereas the second cuticle (protective sheath) is flexible and is often wrinkled.

Larvæ with two sheaths from liquid cultures were kept

under observation in cover-glass preparations, and it was noted that as the

slide became almost dry certain larvæ that were in contact with some solid object discarded the first sheath. This observation was repeated a number of times and indicates that contact with a solid object is necessary to exsheathing, and also probably that a liquid medium is inimical to the completion of the ecdysis. According to Augustine (1), the first ecdysis in *Ancylostoma duodenale* is due to the fact that the larva has increased in size considerably and thus bursts the tightly surrounding sheath. The slight difference in size between first- and second-stage larvæ of *Bustomum phlebotomum* renders other mechanical stimuli necessary for the completion of the first ecdysis, and in the absence of these stimuli the first cuticle is retained.

## BEHAVIOR OF INFECTIVE LARVÆ

### GENERAL BEHAVIOR

As observed in liquid-culture dishes and in hanging-drop preparations, infective larvæ show but a moderate degree of activity at room temperature. Periods of activity and rest alternate, and while at rest the larva is either straight and rigid or somewhat coiled and rigid. The protective sheath is generally visible, because the body is always more or less retracted with-

FIG. 4.—Infective larva. oes., oesophagus; n. r., nerve ring; sh., sheath; int., intestine; g. p., genital primordium

in the sheath. Quiescent third-stage larvæ usually become active when disturbed. A transfer from a culture dish to a slide stimulates them to activity involving rapid movements, which gradually become less intense and ultimately cease entirely.

On slide preparations it was noted that the larvæ commonly exhibit a tendency to come to rest when in contact with some solid object such as debris carried over from the culture medium. Larvæ with the anterior

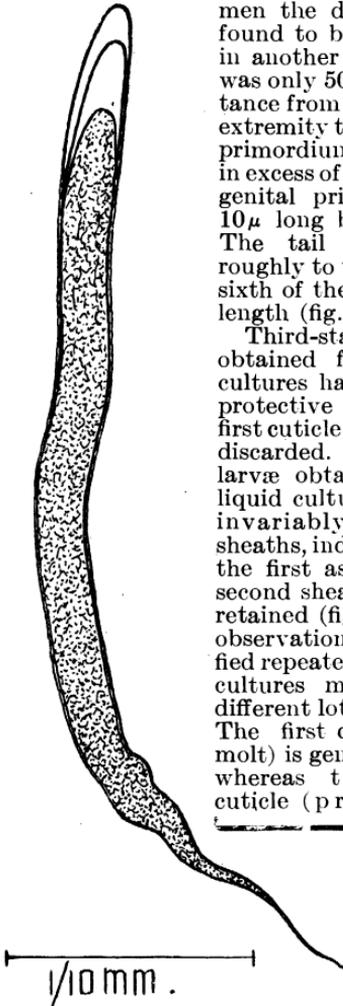
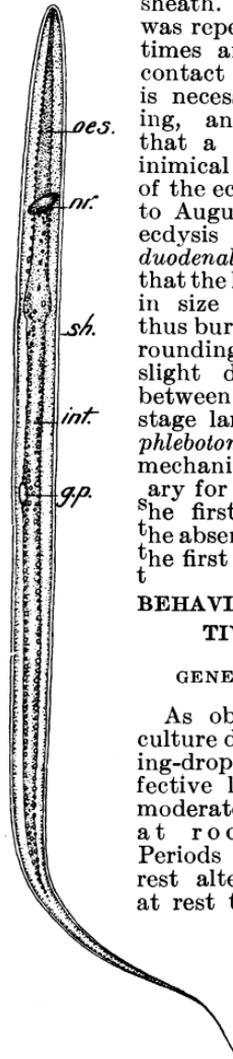


FIG. 3.—Outline drawing of infective larva from liquid culture medium showing two sheaths



portion of the body concealed under some solid object and the rest of the body straight and rigid are usually seen in culture dishes and cover-glass preparations.

Infective larvæ appear generally more tolerant of an unfavorable environment than are preinfective larvæ. Preinfective larvæ are easily killed in culture dishes when bacteria are present in large numbers, the heaviest mortality occurring during the lethargus. But infective larvæ were found in culture media containing massive bacterial growths. While preinfective larvæ can maintain their vitality at a low temperature, they frequently degenerate in a refrigerator, whereas this phenomenon was not observed in infective larvæ, which were kept at 8° C. in water without showing any signs of degeneration. Infective larvæ were also frozen solid for about 15 hours outside of a window, and after being thawed they gradually resumed their normal shape and became active, showing what appeared to be a complete recovery in about 5 hours, whereas preinfective larvæ are easily killed by cold, according to Conradi and Barnett (4). Ransom (12) showed that the preinfective larvæ of *Haemonchus contortus* offer comparatively little resistance to freezing, although infective larvæ could be frozen and successively thawed out many times.

#### REACTION TO HEAT

Under the influence of heat the larvæ exhibit very lively movements. Thus, if the point of a needle heated in a flame or the heated end of a glass rod is brought into contact with the under-surface of a glass slide containing larvæ, they become very active and orient themselves toward the source of the heat, moving very rapidly in that direction. The orientation of the larvæ with reference to the source of heat is unerring, and the writer took advantage of this reaction to concentrate larvæ in the center of a dish in which comparatively few larvæ were present. Holding a hot glass rod against the outer surface of the bottom of a Petri dish caused the larvæ to collect in a circumference around the point to which heat was applied, approaching thither from all directions.

While heat is an unerring source of stimulation to the larvæ, it was noted that larvæ which collected near the edge of a drop of water on a slide, a tendency which they frequently exhibit, do not orient themselves toward the source of heat but merely exhibit feverish activity by vigorous and

spasmodic movements of the body, without turning the cephalic extremity which continues to be in contact with the periphery of the drop.

If too much heat is applied the larvæ cease their movements before they reach the source of heat, coiling up instead and resuming their activities as the heat diminishes.

Khalil (9) showed that the infective larvæ of a number of nematodes are positively thermotropic. He obtained positive results with *Ancylostoma duodenale*, *Necator americanus*, *Ancylostoma ceylanicum*, *Strongyloides stercoralis*, *Galoncus perniciosus*, and *Trichostrongylus douglasi*, and negative results with *Haemonchus contortus*. As *Haemonchus contortus* is not a skin penetrator, Khalil concluded that only skin penetrators are positively thermotropic. Recently Cameron (3) showed that *Monodontus trigonocephalus* is positively thermotropic, although the larvæ do not penetrate the skin under experimental conditions identical with those under which *Ancylostoma ceylanicum* penetrates it. As will be shown later, *Bustomum phlebotomum* does not penetrate the skin under experimental conditions, thus affording further evidence that there is no correlation between positive thermotropism and ability of larvæ to penetrate the skin. In this connection it may be of interest to note that the larvæ of *Nematodirus* are negatively thermotropic according to Cameron's observations (3).

#### REACTION TO STAIN

The reaction of the infective larvæ to stain was studied by allowing a 1 per cent solution of basic fuchsin to run in under cover-glass preparations containing larvæ. As the stain runs in the larvæ are active at first; but that this activity is independent of the stain and is due to the current can be readily shown by running in water under the cover-glass. As the larvæ become enveloped in stain they generally become quiescent but do not absorb the stain. Larvæ were kept under observation for several hours (6 hours in one case and 3 hours in two other cases), but no penetration of the stain into the tissues of the larvæ was observed. The stain readily penetrates the sheath, as is seen distinctly whenever a larva retracts within the sheath, but the writer found no evidence of penetration of the stain beyond that point. The larvæ remained in the stain for hours without loss of vitality. Their vitality while quiescent can be demonstrated (1) by running water

under the cover glass, the current thus set up acting as a stimulant to activity, (2) by applying heat, (3) by watching for spontaneous signs of activity, which usually occur at variable intervals. Throughout this series of observations, involving a number of larvæ, one larva showed evidence of having absorbed some stain, but it had lost its vitality and showed no response to stimulation.

Cameron (3) states that the larvæ of *Monodontus trigonocephalus* when treated with fuchsin absorb the stain rapidly and die, in contrast to the behavior of the larvæ of *Ancylostoma duodenale*, which fail to absorb the stain and escape from their sheaths, which alone become stained, as shown by Looss (10) and by other investigators. Cameron also states: "Goodey (8) showed that this was also true for *Necator* larvæ; but that *Haemonchus* and *Graphidium* which do not penetrate the skin, absorbed the stain and died." He further states that all skin penetrators that have been tested exsheathed, whereas nonskin penetrators have not exsheathed but died when treated with stain. In Goodey's paper to which Cameron refers the only statements concerning reaction of nematode larvæ to stains are the following: "I found that *N. americanus* larvæ come out of their sheaths, as found by Herman and confirmed by Looss, when the drop containing the larvæ and stain is covered with a cover slip. *G. strigosum* and *T. retortaeformis* larvæ did not exsheath." Nowhere in Goodey's paper are there any references to the behavior of *Haemonchus* larvæ in the presence of stain or any reference to the fact that the larvæ of *Graphidium* and *Trichostrongylus* were killed by stains (methyl green and fuchsin).

In the present writer's experiments *Bustomum* larvæ did not exsheath in the presence of fuchsin, nor coil up and die. As will be shown later, there is no necessary correlation between the reaction of larvæ to stains and their ability to penetrate skin.

#### EFFECTS OF DRYING

According to Conradi and Barnett (4), infective larvæ of *Bustomum phlebotomum* are resistant to drought, but according to the writer's observations infective larvæ of *Bustomum phlebotomum* are not resistant to drying. Slide preparations with and without cover glasses containing larvæ were allowed to dry at room temperature for periods varying from one to several hours. After being moistened it was found

that the larvæ were retracted in their sheaths, and although the retracted larvæ absorbed water, as a result of which they gradually filled out the empty spaces in the cuticle, they did not regain their vitality. These observations were made during the winter months in a steam-heated laboratory having a very low humidity content, and were repeated a number of times with similar results. Although the larvæ are not resistant to drying, they can maintain their vitality for a long time in the presence of a small amount of moisture. Thus a solid-culture medium in a covered Petri dish to which water had not been added for about three weeks and which had the appearance of being dry yielded larvæ many of which had retained their vitality and exhibited lively movements after being moistened.

Nematode larvæ exhibit considerable variation in resistance to drying. Ransom (12) showed that the larvæ of *Haemonchus contortus* are highly resistant to drying, and that they can be revived after 35 days' exposure to drying. Looss (10) found that the larvæ of *Ancylostoma duodenale* shrivel up and die when the moisture evaporates from their bodies. Boulenger (2) showed that *Nematodirus* larvæ are resistant to drying, and this observation has been confirmed by Cameron (3).

Goodey (8) confirms Looss's observation for *Necator americanus* and states: "It seems a natural inference to draw therefore that *Necator* and *Ancylostoma* seek the protection afforded by penetration into the skin because if they remained outside and became dry they would perish." The same writer found that the larvæ of *Graphidium strigosum* and of *Trichostrongylus retortaeformis* can withstand ordinary desiccation at room temperature for a few days and revive on the addition of water, behaving in this respect like the larvæ of *Haemonchus contortus* as shown by Ransom (12) and confirmed by Veglia (15). Cameron (3) found that the larvæ of *Monodontus trigonocephalus* offer little resistance to drying, and since these larvæ are not skin penetrators, it is unsafe to attempt to correlate susceptibility to drying with the habit of boring through the skin. The present writer's observations with reference to the behavior of the larvæ of *Bustomum phlebotomum*, which do not penetrate the skin under experimental conditions, lend no confirmation to the view that nonskin penetrators are resistant to drying.

## REACTION TO LIGHT

The ensheathed larvæ of *Bustomum phlebotomum* are positively phototropic, as the following observations will show.

In a 14-day-old culture on a charcoal-and-feces mixture larvæ were found along the walls of the bottle facing the light of a north window, but none was found along the walls of the bottle facing the room. In a 10-day-old culture which was kept in a dark place no larvæ were found along the walls of the bottle, whereas in a culture made from the same lot of worms but kept near the window larvæ were found crawling up the walls of the bottle facing the light. Similar observations made at various times on larvæ in cultures indicate that they respond positively to light by collecting in parts of culture dishes exposed to light and by being absent from shaded portions.

In their light reactions the larvæ of *Bustomum phlebotomum* resemble the larvæ of *Monodontus* (Cameron, 3) and *Haemonchus contortus* (Veglia, 15).

## REACTION TO GRAVITY

Conradi and Barnett (4) noted that the larvæ of *Bustomum phlebotomum* crawl up the walls of culture jars. The present writer's observations bear out this point. Whether this upward movement of the larvæ is a negative geotropism or whether it is merely an expression of their positive phototropism is not clear from the data at hand. The tendency of the larvæ to climb up the walls of culture dishes, and their presumably similar tendency to climb up blades of grass and other objects in nature, is doubtless an adaptation to secure an advantageous position in order to complete their life cycle. Ransom (12) pointed out the adaptation of the upward migration of *Haemonchus* and other strongyle larvæ to the feeding habits of ruminants, which favors the ingestion of the larvæ by the host. Similar tendencies have been observed in most larvæ belonging to the superfamily Strongyloidea, *Syngamus*, according to Ortlepp (11), and *Monodontus*, according to Cameron (3), being exceptions.

## EXPERIMENTS ON SKIN PENETRATION

In 1919 infective larvæ of *Bustomum phlebotomum* were placed on the skin of guinea pigs, the area on which the larvæ were placed having been previously shaved. In the course of several experiments no local skin reaction was

observed in these experimental animals, and intact larvæ were recovered from the skin. Later, experiments were made in accordance with the method devised by Goodey (7). Skin from a 7-day-old rat was stretched on a thin sheet of cork in the center of which was a hole about an inch in diameter, and the cork was floated in physiological salt solution in a glass dish kept in an incubator at a temperature of 37°. A drop of water containing a number of larvæ was placed on the skin. Repeated examinations showed the larvæ on the surface of the skin. The larvæ were sucked up in a pipette and examined from time to time on a glass slide. There was no evidence of their having molted. After the experiment had been in progress several hours the drop of water on the rat's skin containing the larvæ was allowed to evaporate. A drop of distilled water was added to the skin and the larvæ were sucked up in a fine pipette and examined microscopically. They were nearly all active. The skin was then fixed in 70 per cent alcohol and cleared in lactophenol. Microscopic examination of the cleared skin failed to show any evidence of penetration by the larvæ.

Cameron (3) has shown that the larvæ of a related hookworm (*Monodontus trigonocephalus*) do not penetrate the skin under experimental conditions. Since well-known skin penetrators (*Ancylostoma* and *Necator*) penetrate the skin under similar experimental conditions, this method may be considered a reliable test of the ability of nematode larvæ to bore into the skin.

Assuming, therefore, that the results obtained by Cameron (3) with the larvæ of *Monodontus* and the results obtained by the present writer with *Bustomum* give reliable information as regards the inability of the larvæ of these genera to penetrate the skin, it is evident from the data and discussion presented in the foregoing pages that attempts of various investigators to establish correlations between behavior of nematode larvæ and their probable mode of entry into the host is untenable.

## SUMMARY

(1) Under laboratory conditions at a temperature of 70° to 80° F. the eggs of *Bustomum phlebotomum* hatch in about 96 hours. The first-stage larvæ are found in lethargus 24 hours after hatching, and 24 hours later the second lethargus is in progress. After a second lethargus, which lasts at least 24 hours, third-stage larvæ emerge,

the complete cycle of development up to this stage requiring a minimum of 7 days.

(2) In liquid cultures both cuticles are usually retained by the larvæ, whereas in solid cultures the first cuticle is cast off. Observations on the loss of the first skin by infective larvæ from liquid cultures indicate that contact with some solid object is necessary for exsheathing, and that in a liquid medium this process does not ordinarily take place.

(3) Infective larvæ are only moderately active at room temperature, but may be readily stimulated to activity by mechanical factors. These larvæ appear more resistant to an unfavorable environment than do preinfective larvæ.

(4) Infective larvæ are positively thermotropic and orient themselves in such a way that the cephalic extremity becomes directed to the source of heat, toward which the larvæ swim rapidly.

(5) In the presence of a solution of fuchsin the larvæ behave in a manner unlike that observed heretofore in other nematode larvæ. They neither absorb the stain and die nor do they exsheath, but remain alive and apparently unaffected by the stain, which after several hours' contact with the larvæ does not penetrate beyond the sheath or sheaths.

(6) The infective larvæ succumb to desiccation, but can maintain their vitality under conditions which afford a slight amount of moisture.

(7) The infective larvæ are positively phototropic, collecting in the lightest portion of the culture medium. They also crawl up the walls of culture bottles, but whether the latter is to be interpreted as a negative geotropism or is due to the positive phototropism of the larvæ is not certain.

(8) Under experimental conditions the larvæ show no tendency to penetrate the skin.

(9) Attempts made by various writers to correlate the behavior of various strongyle larvæ with their skin-penetrating habits appear to be untenable from the data presented in this paper and from data obtained by others.

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