PARASITISM OF TRICHOPTERA BY BUNODERA MEDIOVITELLATA (DIGENEA: ALLOCREADIIDAE) AND THE ENCAPSULATION RESPONSES

by

JANINE NICOLE CAIRA

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Department of Zoology

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

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ABSTRACT

The incidence of parasitism by Bunodera mediovitellata in Trichoptera in Tin Can Creek was investigated. Larvae of Lepidostoma roafi had a low incidence of infection , while Psychoglypha alascensis larvae had a high incidence of infection. The encapsulation reactions of these Trichoptera larvae to internal Epon implants, and metacercariae of the parasite encysted within the silk glands, were examined in vivo to determine the details of the cellular encapsulation reactions of Trichoptera to foreign objects in both of these sites, and to determine which hemocytes are involved in the reaction.

The hemocytes of larvae of P. alascensis were capable of encapsulating Epon implants within the hemocoele; the reaction took approximately twenty days longer than those of other insects that have been maintained at 20°C. The hemocytes of larvae of P. alascensis are also capable of encapsulating the metacercariae of their natural parasite B. mediovitellata in the silk glands despite the normal absence of hemocytes from the silk gland lumen. The hemocyte capsules around both the non-living and living objects was typical of the 'ordinary encapsulation reaction' described by Salt (1970). The capsule consisted of an inner layer of flattened cells which were densely packed and an outer layer of cells which were not flattened to the same extent as those of the inner layer.

The hemocytes which took part in both capsules around implants and capsules around parasites were flattened elongated hemocytes which probably correspond to plasmatocytes or granulocytes. However, the production of a Summary Fate Map of free circulating hemocytes demonstrated that hemocyte types are stages in the development of a number of lines of hemocyte types. It appears that the hemocytes taking part in the encapsulation reactions belong to two of these lines of hemocyte types.

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In addition I wish to thank Dr. D.R. Brooks for bringing cladistics to U.B.C., and for assisting me with the cladistic analysis of the insect hemocytes. I also thank Mr. L. Veto who patiently guided me through the preparation of my material for electron microscopy. Dr. J. Maze assisted with the statistical analysis, and I thank him.

Many thanks to Dr. M.J. Kennedy for showing me his sampling sites in Tin Can Creek and for allowing me to see his unpublished manuscript on the life cycle of Bunodera mediovitellata. I am also grateful to Dr. G.B. Wiggins for identifying the specimens of Trichoptera.

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I. INTRODUCTION

Despite the protective nature of their tough exoskeleton, insects are often subject to insult by various pathogens. According to Salt (1970), depending on the size and nature of the pathogen, the insect will respond in one of three fashions. Firstly, small individual organisms, such as bacteria and viruses may be dealt with through phagocytosis; 'phagocytes' engulf the particles and thus eliminate them from the hemolymph. Secondly, particles too large to be phagocytised, such as metazoan parasites, are surrounded by a number of blood cells which adhere to the surface of the parasite: the entire structure is termed a capsule, the process is termed encapsulation. Thirdly, clumps of bacteria or other foreign particles may be isolated in a nodule: this process is a combination of phagocytosis and encapsulation.

In his examination of the metazoan parasites of insects, Maupus (1899) was one of the first to distinguish between the terms 'encapsulation' and 'encystment'; he restricted the first term to the reaction of the host, and the second to the activity of the parasite. Since his time, the investigation of the cellular encapsulation reactions of insects to their metazoan parasites has become of interest to two groups of researchers: (1) those wishing to protect economically important insects from their parasites (Free, 1970), for example the silk moth Bombyx sp.

(Sato et al. 1976), and (2) those wishing to eliminate certain important insect pests such as mosquitoes (Andreadis and Hall, 1976), weevils (Bosch and Dietrick, 1959), spruce sawflies (Hawboldt, 1947), and fleas (Chen, 1934).

In order to obtain a broad and thorough understanding of the cellular defense reactions of insects, it is necessary to examine the reactions in as many orders of insects as possible. Unfortunately, the detailed examinations of the encapsulation reactions of insects have centered around the reactions of a limited number of insect orders. The reactions of a number of other insect groups including Trichoptera have never been closely examined.

The metacercariae of the allocreadiid digenean Bunodera mediovitellata Zimbalik and Roytman naturally encyst within the silk glands of Trichoptera larvae (Kennedy, in lit.). This site provides an interesting system in which to examine the encapsulation reaction of an aquatic insect to a natural internal parasite. Although, the encapsulation reactions of Trichoptera to foreign objects, usually located in the hemocoele, have been examined previously (Linstow, 1897; Thompson, 1915; Neuhaus, 1940; Burns, 1961), these accounts are superficial and, as the authors were more interested in the parasites than the host reaction, the details of the capsule are poor.

It is now generally accepted that hemocytes are the main agents of encapsulation of foreign objects in

insects (Salt, 1963). Although it has been suggested that other components including cells of the fat body (Thompson and Thompson, 1921), and connective tissue (Schell, 1952) may be involved in the reaction, Salt (1963) dismisses these citings as structures that have accidently been encapsulated by the hemocytes, or cells that have been mistaken for cells other than hemoctyes.

The study of the encapsulation response of Trichoptera larvae to metacercariae of B. mediovitellata is worthy of attention for several reasons: (1) the encapsulation response of Trichoptera or other aquatic insects to metazoan parasites has not been examined in detail; (2) the hemocyte complex of Trichoptera and the hemocytes involved in the encapsulation reaction have not been examined in detail; (3) the silk glands are an unusual site for the encystment of a digenean, and therefore an unusual site in which to examine the hemocyte response especially since hemocytes are not normally present within the lumen of the gland.

Thus, the purposes of this study were: (1) to confirm the details of the life cycle of B. mediovitellata and investigate the infection of the Trichoptera larvae involved; (2) to examine the encapsulation reaction of these Trichoptera larvae to metacercariae of B. mediovitellata encysted within the hemocyte-free lumen of the silk glands; and (3) to determine which hemocytes take part in the capsule formation. In addition, the reaction of the Trichoptera larvae to non-living implants located

within the hemocoele, was also studied, in order to compare this with the encapsulation response to the parasite in the silk glands.

II. MATERIALS AND METHODS

1. Field Studies

The life cycle and natural infections of Trichoptera were studied by periodic sampling of the fauna in Tin Can Creek, a small stream that flows from the University Endowment Lands through Musqueum Park, and enters the Strait of Georgia near the mouth of the north arm of the Fraser River. Samples were taken with a dip net, at approximately three month intervals beginning in April 1979 and ending May 1981.

2. Experimental and Laboratory Studies

A. Experimental Animals

(i) Trichoptera

Larval Trichoptera were collected from the bottom detritus of the edges of Tin Can Creek with a small dip net. The collections were taken from the stream as it passes through Musqueum Park, on the south side of South West Marine Drive as it meets 41st Avenue, approximately 100 meters downstream from the tunnel into which it flows from underneath a housing development. All samples were transported from the field to the laboratory in four-liter plastic buckets filled with stream water. In the laboratory the buckets were placed in a constant temperature chamber set at 10°C and later sorted into species. The larvae were fed with decaying leaves also collected from the site in Tin Can Creek.

(ii) Clams

Clams (Pisidum sp.) were collected in netfuls of sand from the bottom of Tin Can Creek. The clams were carried to the laboratory in the same buckets as the larval Trichoptera. If the samples were to be kept for a period of time the clams were removed from these buckets and kept separately in aerated buckets with a layer of sand on the bottom.

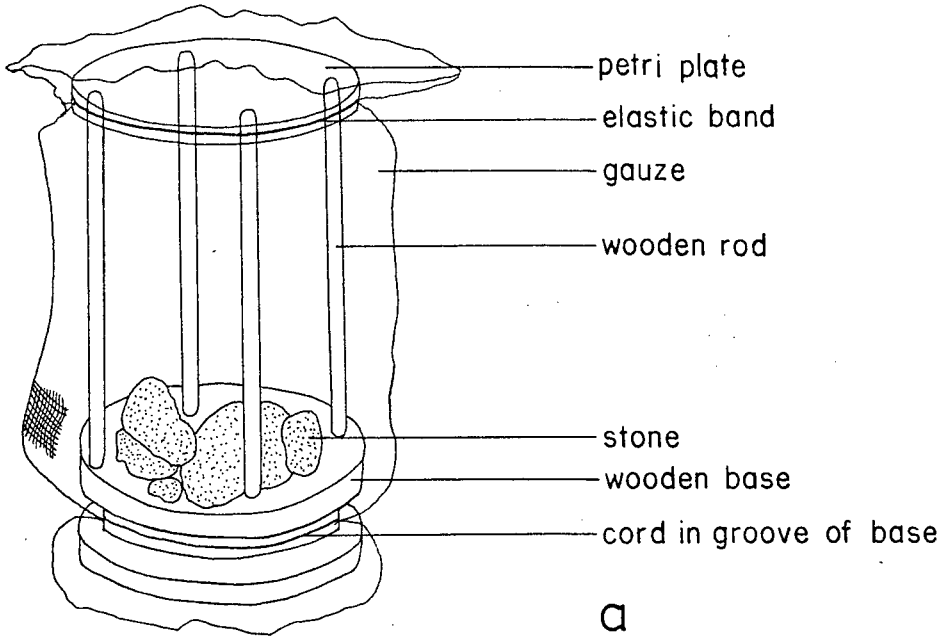
B. Rearing Trichoptera

The apparatus used to rear the Trichoptera larvae to adults is illustrated in Figure 1. This is a design similar to that of Neander (1928); however, it has been slightly modified by the addition of a glass petri-plate viewing lid. In addition, to facilitate the rearing of caddisfly larvae requiring moving water, the whole container was placed in a plastic dish pan. The pan was then placed into a sink and the hose from the cold water tap was clamped to the pan and allowed to run constantly at a medium speed.

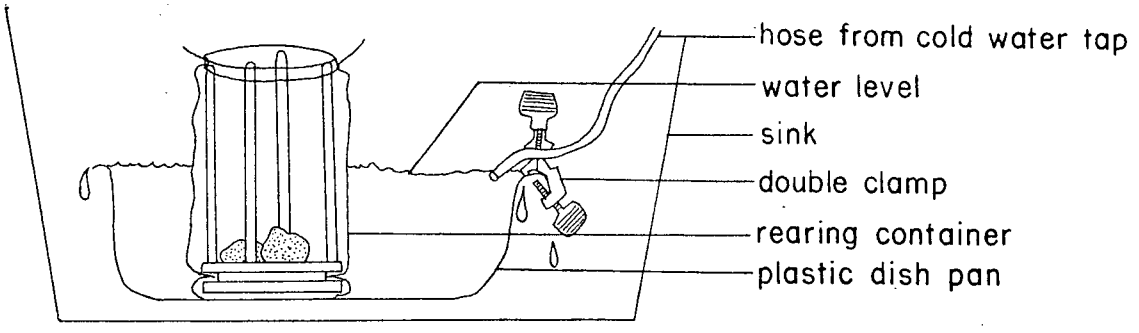
Caddisfly larvae were examined and separated into different morphological types (those possessing a dorsal centering hump on the first abdominal segment and those possessing no hump); twenty larvae of each type were placed in each rearing cage at one time. The larvae were fed ad libitum with decaying leaves collected from Tin Can Creek.

FIGURE 1

Schematic diagrams of the set-up for rearing Trichoptera larvae, (a) rearing chamber, (b) rearing chamber set up in moving water system.


petri plate
elastic band
gauze
wooden rod
stone
wooden base
cord in groove of base

a


hose from cold water tap
water level
sink
double clamp
rearing container
plastic dish pan

b

The rearing containers were visually inspected at least biweekly. Imagos seen clinging to the cheesecloth at the top of the containers were placed in a solution of 70% ethanol and sent to Dr. G.B. Wiggins at the Royal Ontario Museum in Toronto for identification.

C. Statistical Analysis

In order to determine if the number of cysts per individual was affected by the size of the case, species of Trichoptera, or date of collection, an analysis of variance using the U.B.C. Anovar program (Greg and Osterlin, 1978) was applied.

D. Experimental Infection

Eight larvae of each of the species of caddisfly, measuring between 10 and 15 mm. in length (including the case), were removed from their cases and placed in dechlorinated water in wells of well-plates set up under a stereo dissecting microscope. Clams in small petri-dishes were opened with microforceps and cercariae were removed from the clams by breaking the redial membranes. Ten to 20 cercariae were pipetted into each of the wells containing Trichoptera larvae. These larvae were visually examined for penetration of cercariae over a period of approximately one hour. After three hours the larvae were dissected and examined for cercariae both in the body cavity, and in particular within the silk glands.

As the animals being infected were from a natural population, and therefore not necessarily uninfected, eight non-experimental field caught larvae of each species were isolated, dissected and also examined for cercariae within the body cavity and silk glands.

E. Silk Gland Dissection Procedure

Trichoptera larvae in dechlorinated water were dissected under a stereo dissecting microscope using two pairs of microforceps. One pair was used to firmly hold the prothorax, while the other pair was used to grasp the posterior portion of the head and pull it sharply forward and off. Because the silk glands are attached in this region of the head, they were usually pulled out intact, along with the head.

F. Metacercarial Description

Five metacercariae were teased from their capsules in P. alascensis and removed from their cysts using two fine dissecting needles. The excysted metacercariae were prepared according to the protocol of Chubb (1962) and stained with Erlich's hematoxylin. Drawings were done with the aid of a drawing tube. Measurements were done using an ocular micrometer.

G. Histology

The silk glands of Trichoptera containing metacercariae were embedded in wax and stained with Erlich's hematoxylin

and eosin Y according to the method described in Culling (1963). Sections of metacercariae within the silk glands of the two species of Trichoptera were compared and the following characteristics noted:

(1) general appearance of the cercaria

(2) position of cercaria within the gland

(3) damage to the gland

(4) host reaction to the cercaria.

H. Melanin Test

Ten paraffin sections were prepared from each of two pigmented metacercariae collected on July 28, 1979. Ten of these sections (five from each metacercaria) were treated with the Ferrous Iron Technique of Pearse (1972), a technique specific for staining melanin. This test stains melanin dark green, while the background remains faint green or colourless.

The remaining ten sections were treated with the melanin-specific bleach, potassium permanganate followed by oxalic acid (Pearse, 1972). The bleached sections were then treated with the Ferrous Iron Technique for melanin.

I. Electron Microscopy

Metacercarial cysts to be examined under the electron microscope were prepared according to the protocol of R. Dean (personal communication). Owing to the dense nature of the cysts however, the protocol was altered to

increase penetration. One drop of Photoflow diluted 1:100 was added to the fixation and wash solutions to reduce the surface tension around the cysts. All fixation, wash, dehydration with propylene oxide, and infiltration times were extended to overnight. The cysts were embedded in Spurrs, a low viscosity resin. The fixation, wash, dehydration with propylene oxide, infiltration and embedding were carried out under vacuum. The entire protocol used is given in Appendix 1.

Both thick (4u) sections and thin (approximately 200Å) sections were cut using a Sorval MT-1 ultramicrotome equipped with a glass knife. Thick sections were stained with a solution of methylene blue in 1% borax which had been sitting for some time and therefore had acquired polychrome staining qualities. Thin sections were placed on grids coated with carbon. These sections were stained with lead citrate and examined at 60 kv. with a Zeiss EM-10.

J. Implantation Procedure

Each of six caddisfly larvae was placed in a small vial attached to a CO_2 tank for 4.5 minutes and thus anesthetized. The larva was then removed from its case and attached with plasticine to the bottom of a small weighing dish filled with dechlorinated water. A small longitudinal incision was made on the right dorsal side of the first to third abdominal segments of the animal, and an Epon 812 sliver measuring approximately 0.2 x 1.00 mm,

was implanted with microforceps into this area such that it was lying free within the hemocoele. The larva was then returned to its case and placed in a 300 ml. preparation dish containing decaying leaves and dechlorinated water. The larvae were kept aerated in a constant temperature chamber at 10°C.

After either seven or 35 days, each of three larvae containing implants was removed from its case and anesthetized for ten minutes as above. The area of the incision, (recognizable by the pigmentation of the wound healing reaction at the site of the incision of the integument) was then slit with a razor tool and the hemocoele examined for the implant. Implants found lying free within the hemocoele at this time were immediately placed into fixative and embedded in Epon 812 according to the procedure of R. Dean (personal communication). The Epon 812 blocks were sectioned at 4u with a Sorval MT-1 ultramicrotome equipped with a glass knife. Sections were stained with methylene blue (possessing polychromatic staining ability) in 1% borax.

K. Hemocyte Examination

Hemocytes were heat fixed within the insect by placing the larval Trichoptera in a beaker of 60°C water for five minutes. Immediately after fixation, hemolymph was removed from the abdomen of the insects using a finely drawn glass pipette. The hemolymph was then smeared on a glass slide and allowed to air dry. Each slide was prepared and stained

with Giemsa according to the protocol of Arnold and Hinks (1979). The dry stained smears were covered with permount and #1 coverslips. Each slide was scanned under emersion oil for as many different hemocytes as possible. Photographs of these cells were taken using a camera adapted to a microscope and Ektachrome Tungsten 50 ASA film.

The Wagner method of quantitative parsimony analysis (cladistic) (Farris, 1970) was used to analyze the data. This method produces a branching diagram (cladogram) of the shortest rooted tree for the data set. A list of the characters used to construct the data matrix is given below:

A- area of cell (nucleus area + cytoplasm area)

B- nucleus area

C- cytoplasm area/nucleus area

D- position of nucleus

E- shape of cell

F- compactness of cytoplasm

G- compactness of nucleus

H- cytoplasm granulation

I- cytoplasm vacuolation.

Only those cells whose cytoplasm and nucleus were readily distinguishable were used in the analysis. For those characters involving area measurments (A,B,C) photographs of each cell were transposed to 1 $mm.^2$ graph paper and the number of squares covered by the cytoplasm and nucleus of each cell was counted. Cells were arranged in ascending

order of area and sequential pairwise comparisons of percentage difference was made. Only if this difference was 15.5% or greater was a new character state assigned to the cell. For example, the measurements for the hemocyte cytoplasm area were 12, 13, 16, 18, 18, etc. (mm^2). The percentage difference between 12 and 13 is approximately 7% therefore these cells are coded within the same character state (eg. 0). The difference between 13 and 16 however, is 19% therefore 16 represents a new state of the cytoplasm area character (eg. 1).

III. RESULTS

A. Trichoptera Larvae Involved and Life Cycle

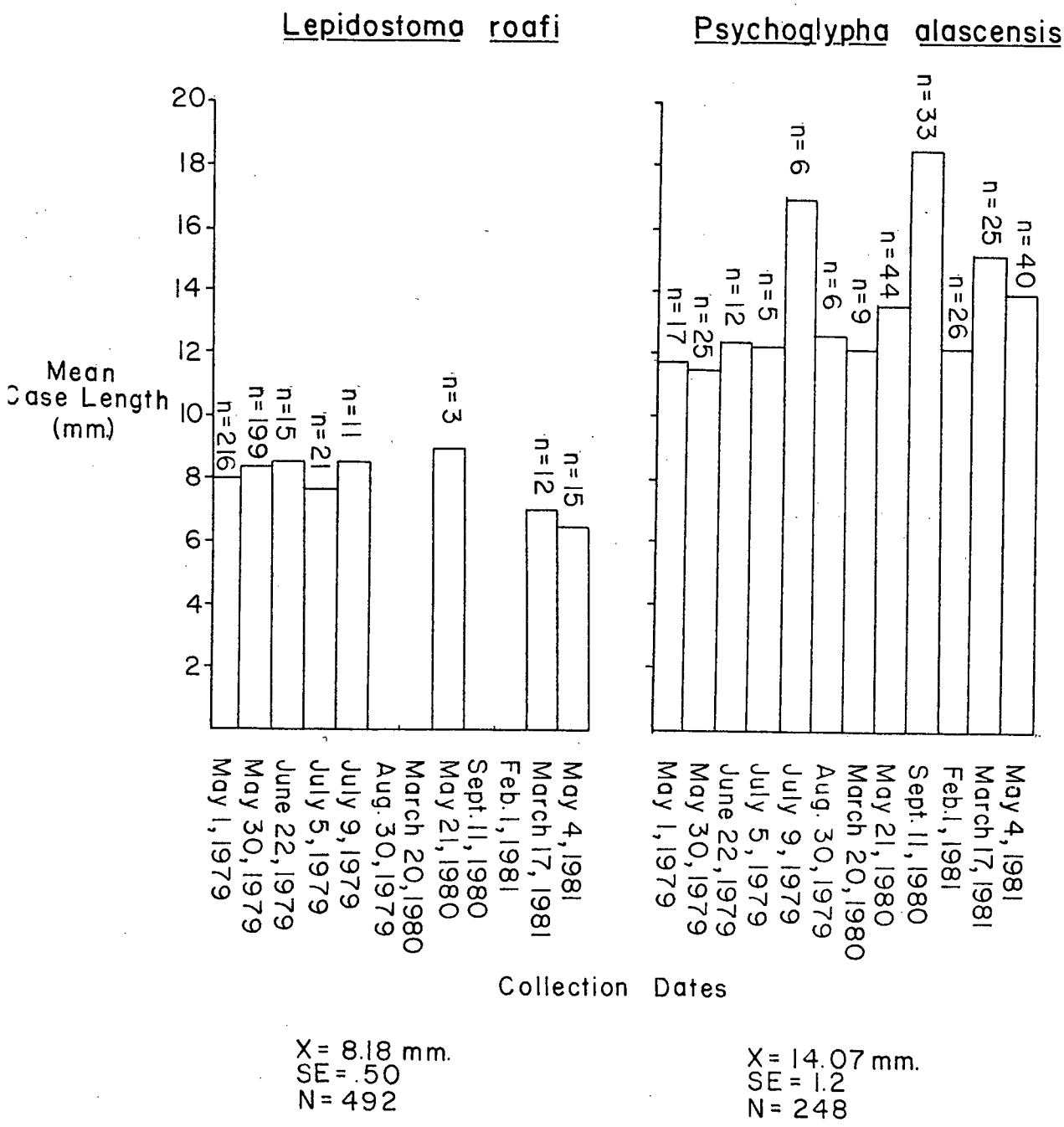
Twelve periodic samples of Tin Can Creek consistently demonstrated the presence and coexistence of two morphologically distinct types of caddisfly larvae. The first type of larva was distinguished by the presence of a median dorsal hump on the first abdominal segment in addition to a lateral hump at each side of this segment. The second type of larva possessed only the two lateral humps.

Case measurements indicated a size difference between the two types of Trichoptera larvae. Measurements of the cases of 248 larvae possessing the dorsal centering hump had a mean length of 14.07 ± 1.19mm.; however, case measurements of 492 larvae possessing no median dorsal hump had a mean length of $8.18 \pm .50$ mm. A histogram demonstrating this size difference for the twelve sampling periods is given in Figure 2.

Larval determination showed the smaller species to belong to the Lepidostomatidae, and the larger to the Limnephilidae. Individuals of the two types were placed separately into rearing containers, in order to obtain adults for identification; four adults of the smaller species (1 female, 3 males) and one male of the larger species emerged. Dr. G.B. Wiggins identified the smaller species as Lepidostoma roafi (Milne)

FIGURE 2

Mean case lengths of the two species of Trichoptera over the twenty-four month collecting period.



X = 8.18 mm.
SE = .50
N = 492

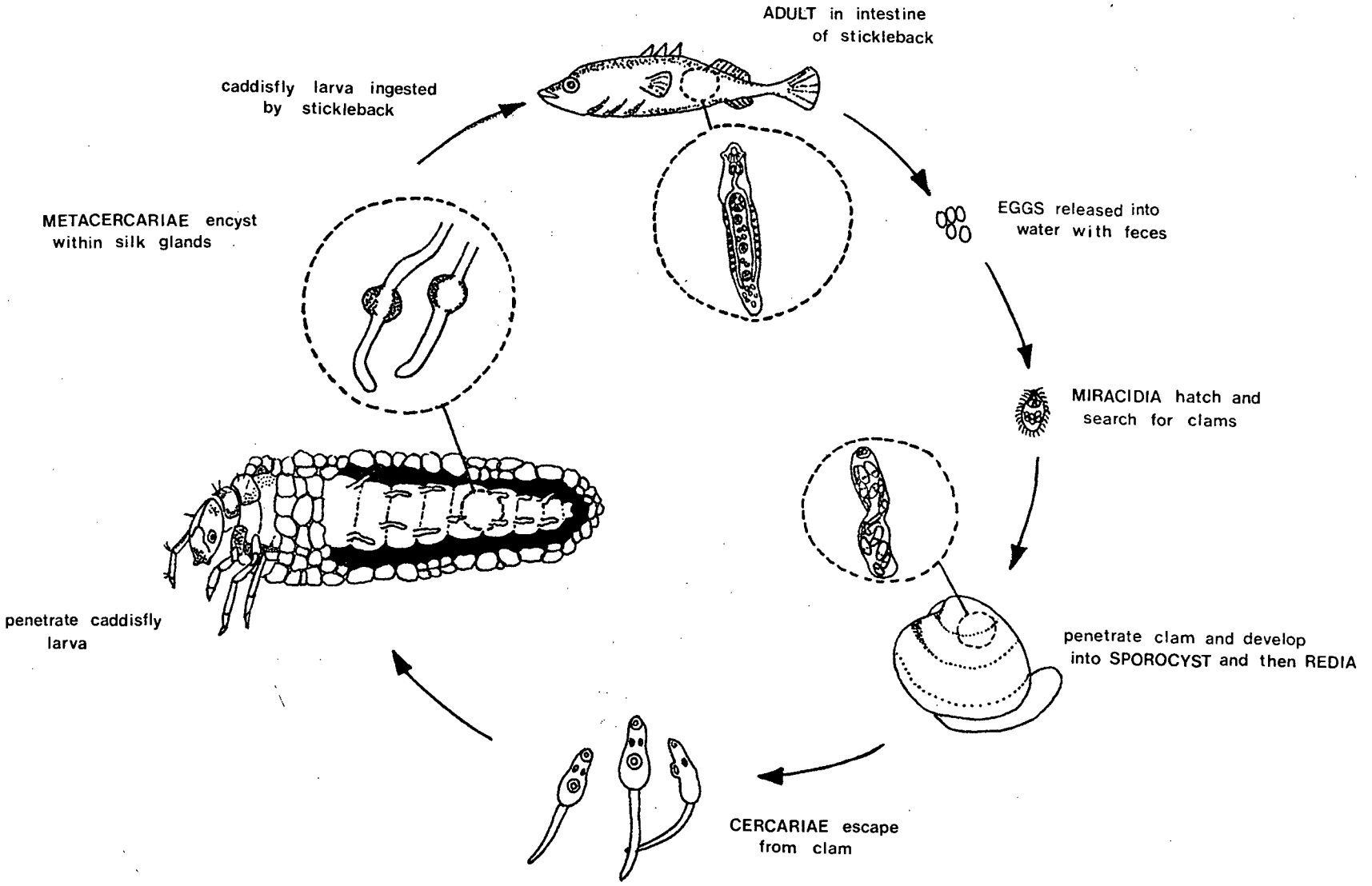
X = 14.07 mm.
SE = 1.2
N = 248

(Lepidostomatidae) and the larger species as Psychoglypha alascensis (Banks) (Limnephilidae).

In Tin Can Creek the larvae of both species of Trichoptera act as second intermediate hosts in the life cycle of the trematode Bunodera mediovitellata (Allocreadiidae). The adult of B. mediovitellata inhabits the intestine of the three-spined stickleback, Gasterosteus aculeatus L. Eggs of the parasite are released from the adult in situ, and are passed out with the feces of the fish. A miracidium emerges from each egg and enters a clam of the genus Pisidium. A sporocyst develops within the gill lamellae of the clam, and later as a redia within the digestive gland. Cercariae are released from the redia. Each cercaria leaves the clam and is swept into the case of a Trichoptera larva via the circulation of water generated by abdominal ventillation movement. The cercaria then attaches to the abdomen of the larva, penetrates through the integument and eventually reaches one of the silk glands. The metacercaria then enters the silk gland, encysts and develops within the cyst. When the Trichoptera larva is ingested by a stickleback, contact with the digestive juices of the stomach releases the metacercaria from its cyst. The metacercaria then moves to the intestine of the fish and develops into the adult. The life cycle of B. mediovitellata as described by Dr. M.J. Kennedy (in lit.) was thus confirmed and is diagrammed in Figure 3.

FIGURE 3

The life cycle of Bunodera mediovitellata.


ADULT in intestine
of stickleback
caddisfly larva ingested
by stickleback
METACERCARIAE encyst
within silk glands
EGGS released into
water with feces
MIRACIDIA hatch and
search for clams
penetrate caddisfly
larva
penetrate clam and develop
into SPOROCYST and then REDIA
CERCARIAE escape
from clam

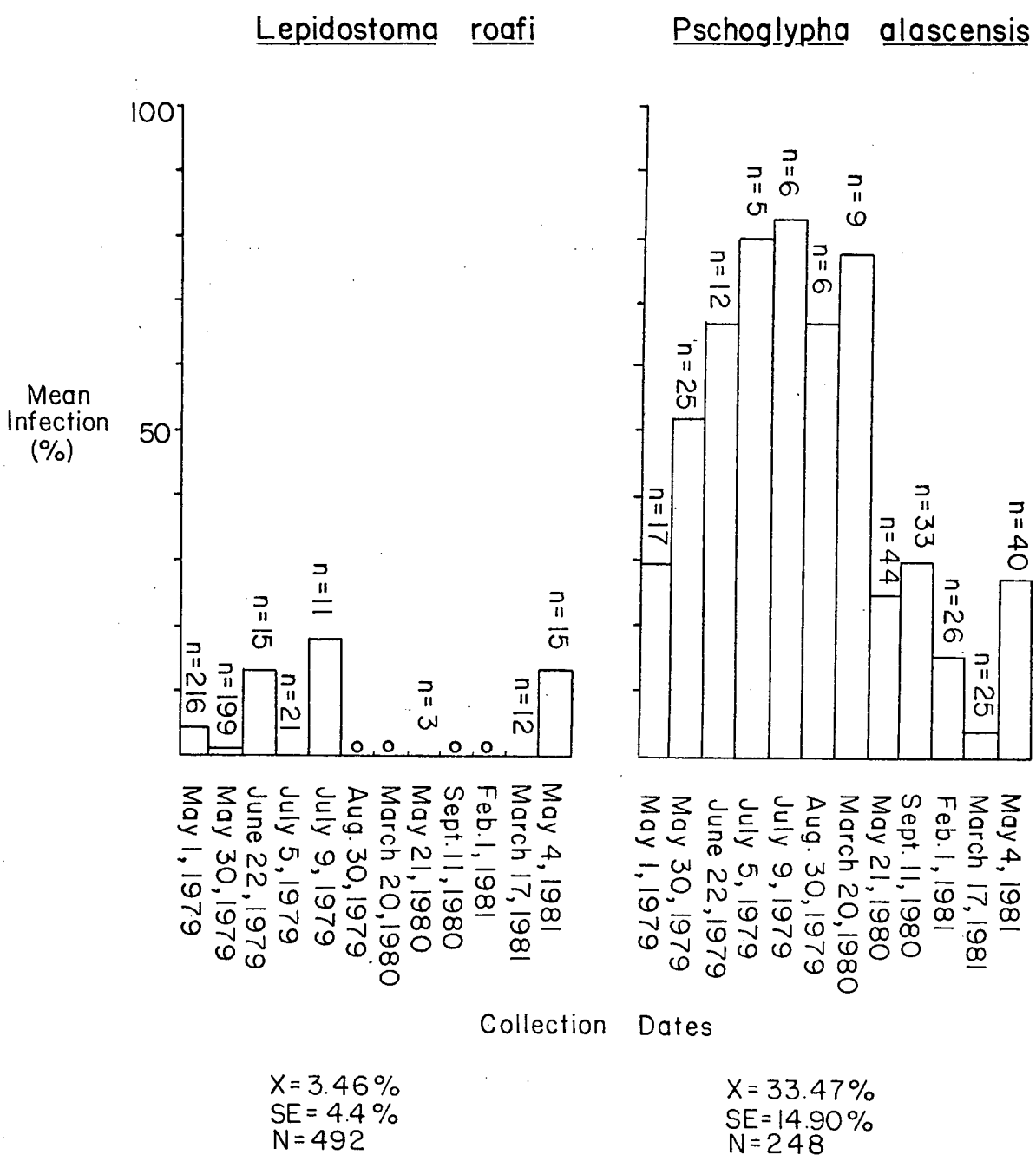
B. Natural Infections

Detailed dissections of 25 individuals of each species of Trichoptera collected May 30, 1979 demonstrated the presence of the metacercariae of B. mediovitellata within the silk glands of both species of caddisfly larvae; metacercariae of the trematode were found nowhere else in the body of either species of caddisfly. In addition, one unencysted progenetic metacercaria was discovered floating within the abdominal hemocoele of a specimen of P. alascensis collected September 11, 1980; it was identified by Dr. D.R. Brooks as an undescribed species belonging to the family Waretrematidae, with morphology similar to that of the members of the genus Megasolena.

Examination of the silk glands of a number of individuals of P. alascensis and L. roafi indicated that there was a difference in incidence of infection between the two species. The mean percentage incidence of infection (at least one metacercaria per individual) of 242 individuals of P. alascensis was $33.7 \pm 14.90\%$; however, the incidence in 497 individuals of L. roafi was $3.34 \pm 4.4\%$. The incidence of infection in both species of Trichoptera over the two year collection period is illustrated in Figure 4. The incidence of infection in L. roafi was at its lowest (0-2%) on May 30, 1979, July 30, 1979, May 21, 1980, and March 17, 1981; it was highest (18%) on July 9, 1979. The incidence of infection in P. alascensis was at a low of 4% on March 17, 1981. The

FIGURE 4

Incidence of parasitism by Bunodera mediovitellata in the two species of Trichoptera. o= no larvae of Lepidostoma roafi collected on that date.



X = 3.46%
SE = 4.4%
N = 492

X = 33.47%
SE = 14.90%
N = 248

highest incidences of infection in P. alascensis (75%) were on July 5, 1979, July 9, 1979 and March 20, 1980. Samples were not taken in June or July of 1980, therefore, it is difficult to assess whether or not this high percent parasitism in the summer is a repeated trend.

Many of the Trichoptera had multiple infections of more than one metacercaria per individual. The infections ranged from one to ten metacercariae per individual in P. alascensis (max. 5 cysts per gland) and from one to four in L. roafi (max. three cysts per gland). The results of the analysis of variance (Table 1) indicated that date of collection and the species of Trichoptera, both had a significant effect on the variance in the number of cysts per individual ($P<.05$). Size of the case, however, did not appear to have a significant effect (P .05).

C. Experimental Infections

In order to determine if the difference in incidence of infection between the two species of Trichoptera was a result of differential penetration of the caddisfly larvae by the cercariae, penetration was examined experimentally. Enough swimming cercariae were obtained to examine penetration of five larvae of each species of Trichoptera. Within the one hour observation period, three cercariae were seen to drop their tails and penetrate one larva and one cercaria to drop its tail and penetrate another larva of the five individuals of L. roafi exposed to them. Upon dissection, all of the

Table 1. Analysis of the effect of date of collection, species of Trichoptera and size of case on the variance of the number of metacercarial cysts per larva.

Source	D.f.	Mean Square	F value	F Probability
Date	11	6.09	3.19	0.0037*
Species	1	13.55	7.09	0.0109*
Size	18	2.33	1.22	0.2929

*= Significant at .05 level.

remaining three larvae, in addition to the five non-experimental field caught larvae examined were found to contain no metacercariae either in the body cavity or within the silk glands.

Two cercariae were seen to drop their tails and penetrate one larva and one cercaria to drop its tail and penetrate another larva of the five individuals of P. alascensis exposed to the cercariae. Dissection showed the remaining three exposed larvae to contain no cercariae within the body cavity or silk glands. Of the five non-experimental field caught larvae of P. alascensis dissected and examined for cercariae, one contained two metacercariae within one silk gland; the remaining four contained no metacercariae.

The numbers of cercariae entering each species of Trichoptera were too small to analyze statistically. However, as cercariae of B. mediovitellata were seen to penetrate individuals of both species of Trichoptera, it seems likely that the difference in the incidence of infection between the two species of caddisfly larvae is not due to differential penetration by the cercariae.

D. Metacercarial Cysts from the Two Species of Trichoptera

It seems likely that both species of Trichoptera serve as the normal intermediate hosts for the metacercariae of B. mediovitellata although there was a difference in incidence of infection. To check that both hosts are

able to support the parasite life cycle, metacercariae encysted within the silk glands were taken from both larvae of P. alascensis and L. roafi, sectioned and examined. The results of this examination are summarized in Table 2. Between the two species of Trichoptera there appeared to be no difference in size of the parasite, its location within the silk gland, the host's reaction to the parasite or the appearance of the parasite: metacercariae remained alive in both host species. A metacercaria from a larva of L. roafi and one from P. alascensis are shown in Figure 5.

This preliminary study demonstrated no difference between metacercariae encysted within the two species of Trichoptera. Because the larvae of P. alascensis were usually more abundant, and had a much higher incidence of infection than larvae of L. roafi, individuals of P. alascensis were used throughout the rest of the study.

E. Metacercarial Description

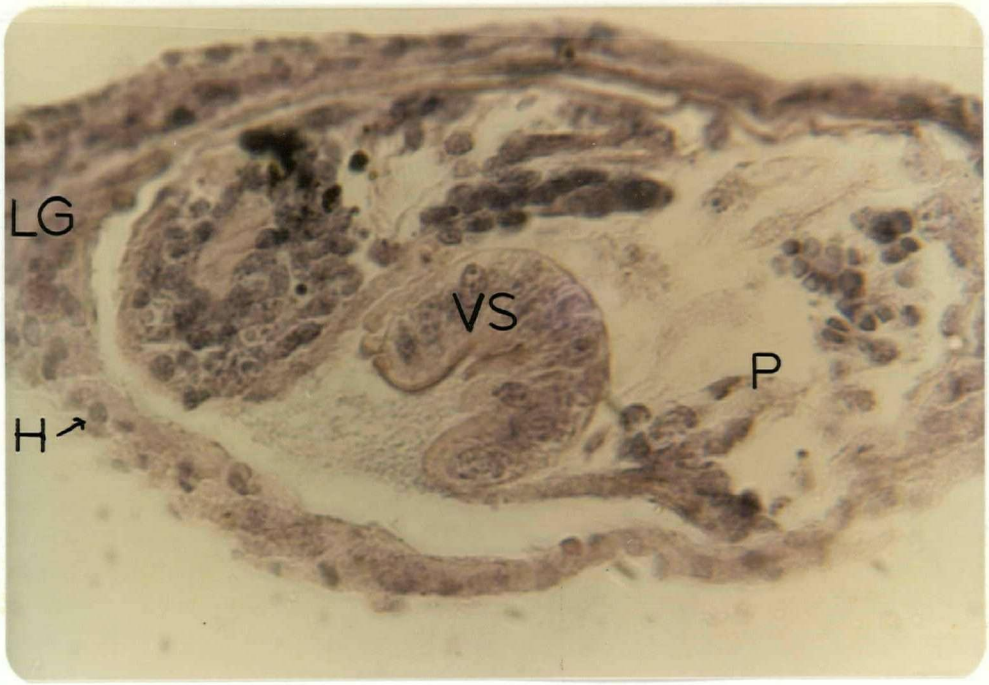
Since a knowledge of the anatomy of the metacercaria is of some use for aging the various metacercarial cysts, this stage was studied and is described for the first time: all measurements are in micrometers. Body length 311 (min. 275-max. 340). Body width 184 (min. 137-max. 231). Cuticle aspinose. Anterior sucker 66 (min. 48-max. 75) by 71 (min. 55-max. 87) bordered laterally by two muscular papillae and with four dorsal papillae. No traces of

Table 2. Comparision of encystment and encapsulation of metacercariae of Bunodera mediovitellata between two species of Trichoptera.

Characteristics	Psychoglypha alascensis	Lepidostoma roafi
Size of metacercariae (diameter across cyst)	120- 175 x 120- 170u	130- 195 x 119-150u
General appearance of metacercariae	Healthy	Healthy
Position of metacercariae within the silk gland	Directly within lumen of gland	Directly within lumen of gland
Damage to the gland	Epithelial layer of gland wall degenerating	Epithelial layer of gland wall degenerating
Host reaction to metacercariae	Hemocytes visible around cyst	Hemocytes visible around cyst

FIGURE 5

Longitudinal sections of metacercariae encysted within silk glands of Trichoptera. (a) Cyst within silk glands of Lepidostoma roafi (Hematoxylin and eosin Y, 675x), (b) Cyst within silk gland of Psychoglypha alascensis (Hematoxylin and eosin Y, 675x). H= hemocyte, LG= lumen of the silk gland, P= parasite, VS= ventral sucker of metacercaria.



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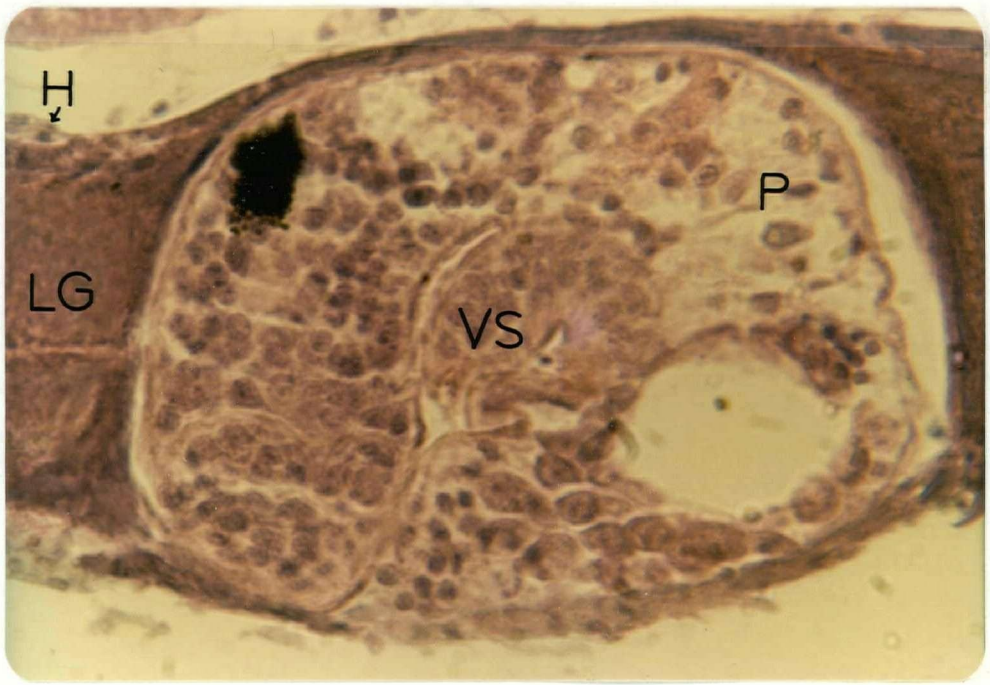

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penetration glands observed. Remains of eyespot pigment clearly visible on either side of the esophagus between pharynx and bifurcation of the gut. Stylet seen in one metacercaria. Prepharynx 8 (min. 5-max. 11) long. Pharynx 29 (min. 25-max. 31) long by 32 (min. 24-max. 44) wide. Esophagus 31 bifurcating at center point of acetabulum. Intestinal caeca thick, extending into posterior one-quarter of body. Acetabulum in anterior one-half of body 69 (min. 63-max. 90) by 67 (min. 50-max. 88). Two round testes tandem, anterior testis 17 by 17, posterior testis 21 by 20. Ovary posterior to acetabulum 10 by 11 Ootype region visible. Uterus not visible. Vitelline follicles extracaecal ranging from oral sucker to tips of caecae. Excretory vesicle not visible. One of the excysted metacercariae is illustrated in Figure 6.

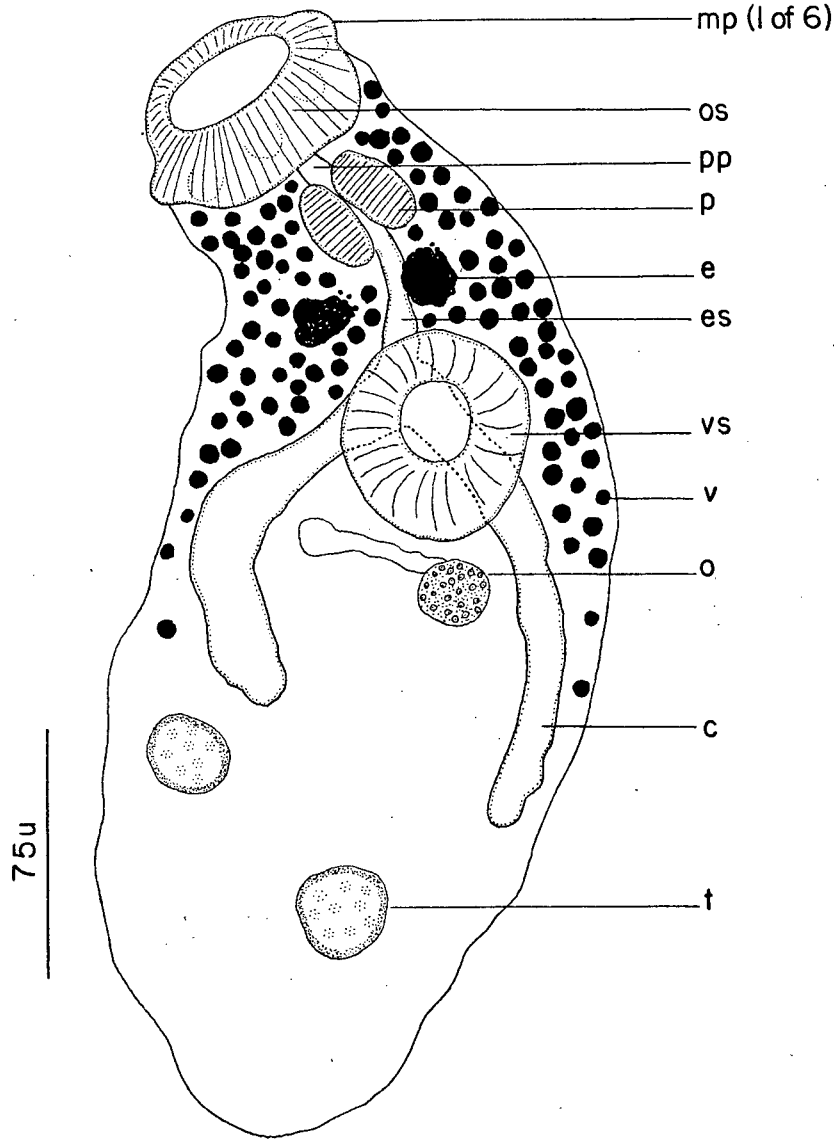
F. The Silk Glands: Normal Structure

Since one of the aims of this study was to examine the cellular response of the Trichoptera larvae to the presence of a parasite encysted within their silk glands, it was first necessary to examine the normal construction and histology of uninfected glands. Changes due to parasitism of silk glands could then be detected and described in relation to the normal morphology of the glands.

Examinations of whole unfixed silk glands indicated

FIGURE 6

Excysted metacercaria of Bunodera mediovitellata. Drawing done with the aid of a drawing tube. c= intestinal caeca, e= eye spot pigmentation, es= esophagus, mp= muscular papilla, o= ovary, os= oral sucker, p=pharynx, pp= prepharynx, t= testis, v= vitellaria, vs= ventral sucker.


mp (1 of 6)
os
pp
p
e
es
vs
v
o
c
t
75u

that these glands were long sacs constricted approximately 1 mm. from the anterior end, and blind at the posterior end. When examined under surface lighting with a stereo dissecting microscope, the wall of the glands appeared to be composed of large, tightly packed hexagonal cells.

Longitudinal sections of uninfected silk glands fixed in Bouin's showed the glands to be tubular with a single layered epithelium and a lumen approximately 50-70u in diameter. The silk within the lumen appeared as a homogeneous amorphous substance. Of the 15 uninfected glands longitudinally sectioned, no hemocytes were seen in the lumen of any of the glands. The epithelial cells of the gland were 15-25u in height, and there appeared to be a gap in the epithelial layer corresponding to an intercellular space every 90-170u (arrows in Figure 7). The gaps in the epithelial layer of one side of the gland corresponded to the center of the uninterrupted areas of the outer side of the gland, which suggested the close packing of hexagonal cells.

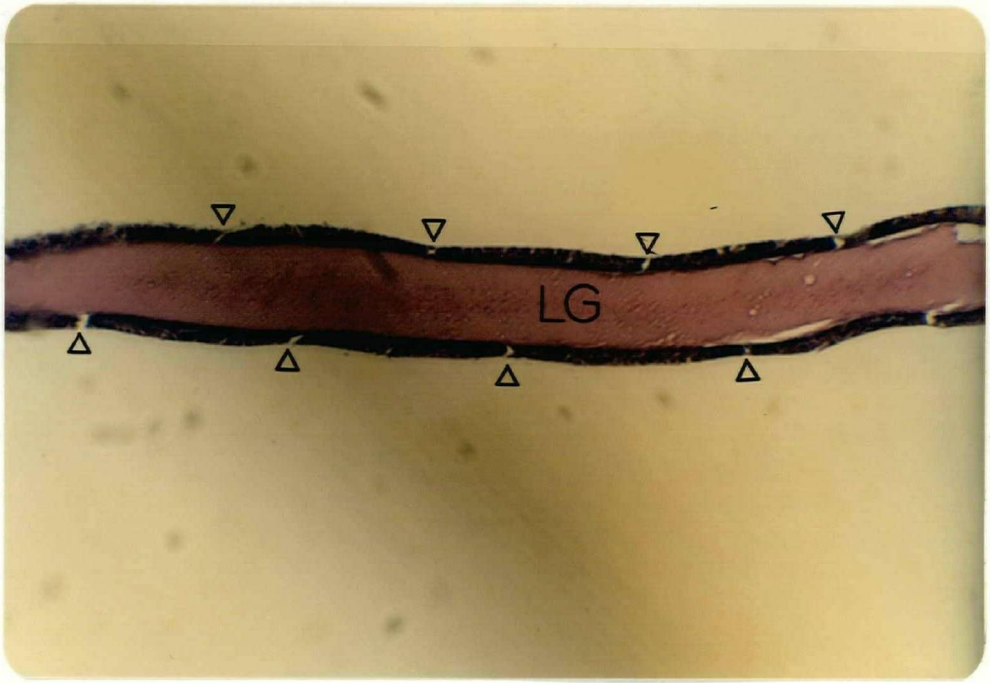
A longitudinal surface-section of the wall of the silk gland also indicated hexagonal cells with large polymorphic nuclei (Figure 8). These nuclei, when seen in section appeared variable in shape and diffuse (Figure 9). A schematic diagram of a portion of a silk gland is given in Figure 10. Note that a cross section of this gland would show intercellular gaps in the epithelium of the gland at the arrows in Figure 10.

FIGURE 7

Longitudinal section of an uninfected silk gland of Psychoglypha alascensis. Arrows indicate intercellular spaces. E= epithelial cells, LG= lumen of the silk gland. (Hematoxylin and eosin Y, 200x).

FIGURE 8

Surface view of a silk gland of Psychoglypha alascensis demonstrating hexagonal epithelial cells with polymorphic nuclei. E= epithelial cell, NE= polymorphic nucleus. (Hematoxylin and eosin Y, 675x).



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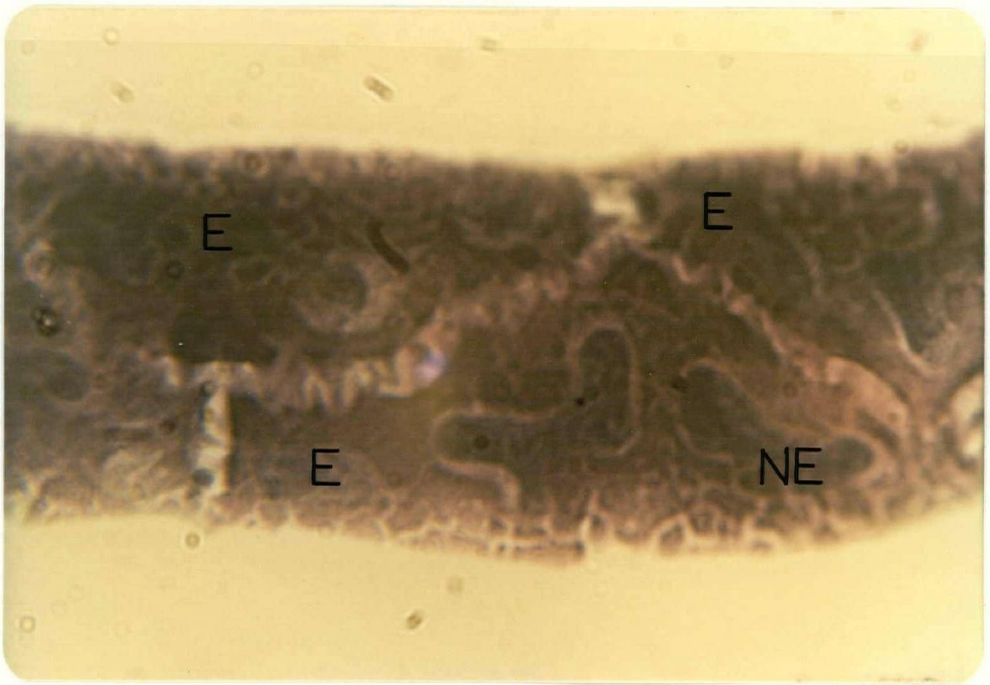

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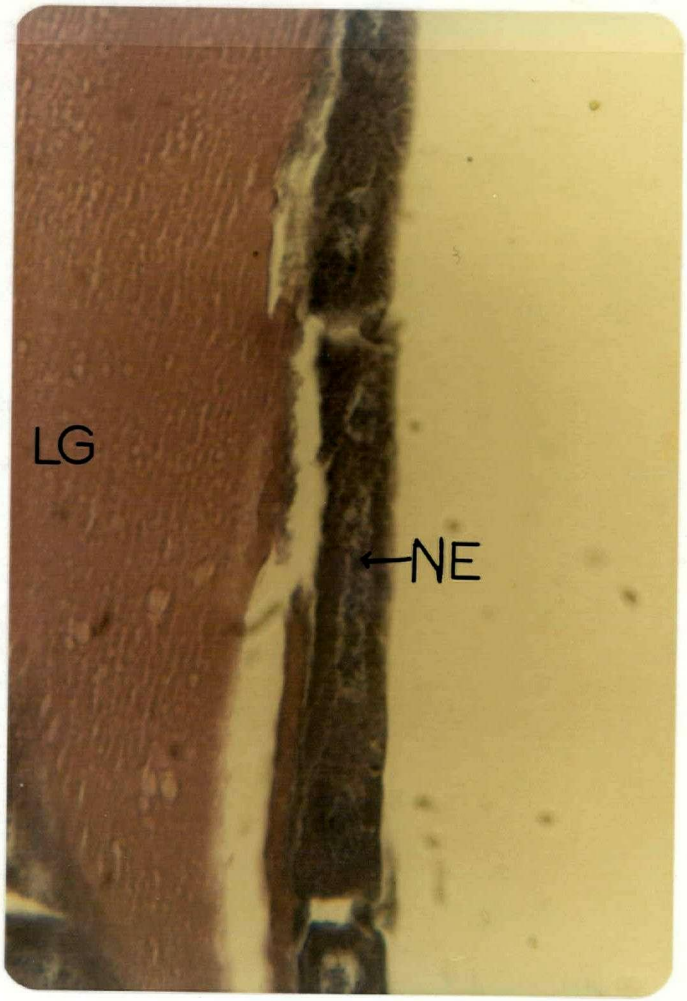
FIGURE 9

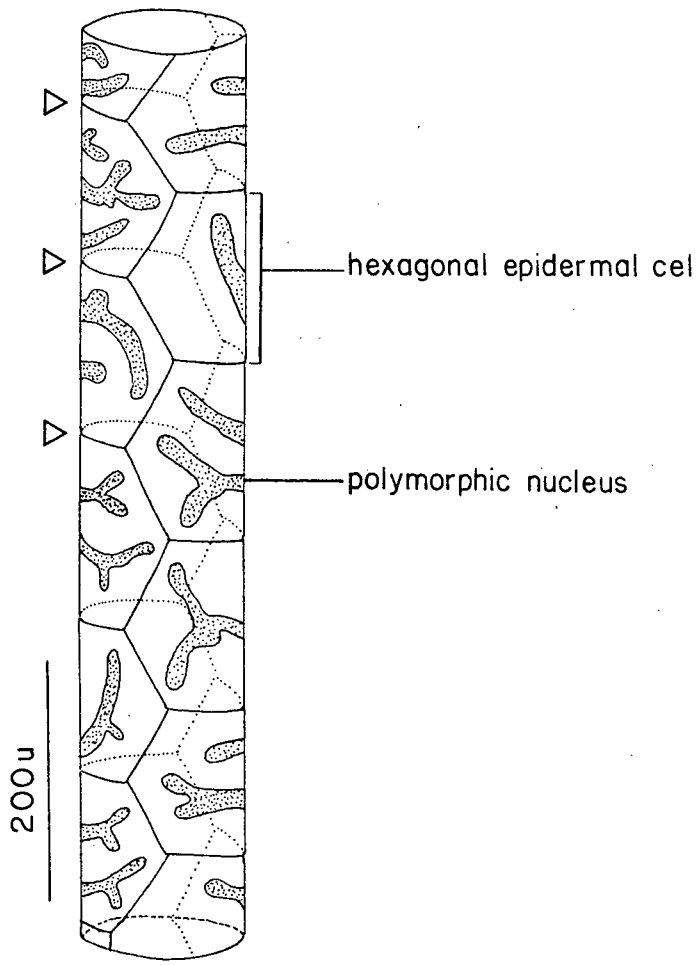
Magnified view of a longitudinal section through a silk gland of Psychoglypha alascensis demonstrating diffuse nature of the epithelial cell nucleus. LG= lumen of silk gland, NE= epithelial cell nucleus. (Hematoxylin and eosin Y, 675x).



9

FIGURE 10

Schematic diagram of the construction of a silk gland. Arrows indicate junctions of epithelial cells, seen as gaps in longitudinal section (Figure 7). Nuclei have been marked in only in the foreground of the diagram.


hexagonal epidermal cell
polymorphic nucleus
200u

G. The Hemocyte Complex

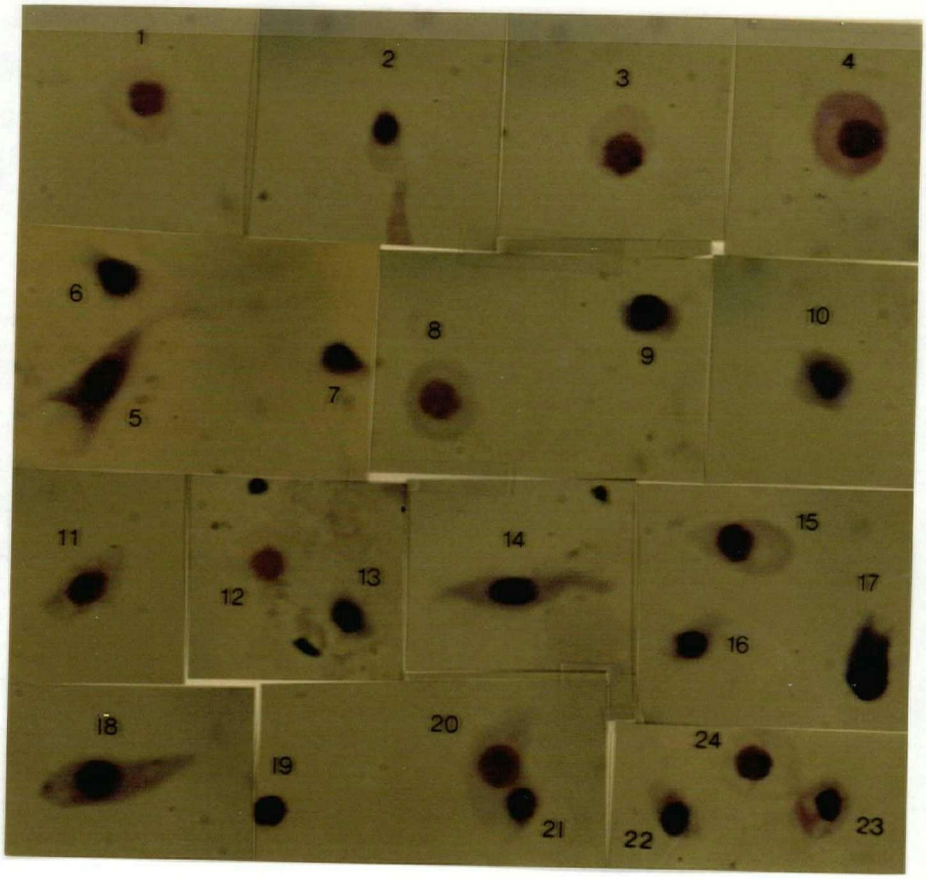
Before examining the hemocyte response of P. alascensis to a parasite it was necessary to examine the complex of free-circulating hemocytes of uninfected larvae. Once the morphology of the free-circulating hemocytes is established it should be possible to compare them with those taking part in the encapsulation response and attempt to determine (i) how the hemocytes change during the encapsulation response, (ii) which of the free hemocyte types take part in the reaction, and (iii) if any hemocytes not seen in the hemocyte smears take part in the encapsulation response.

Figure 11 depicts the 24 morphologically distinct hemocytes found by scanning hemocyte smears. A cladistic analysis was done on these cells to detect any pattern of relationship present among them. The states for each character, in addition to the data matrix constructed for these hemocytes are given in Table 3. The nuclei of the hemocytes observed had a range in size of 3-16u, while the cell length ranged from 5-41u. It will be seen that cells #9 and 21 coded with the same character state sequence, and therefore represent the same 'type' of hemocyte. All of the remaining 23 hemocytes possess a unique coding sequence and therefore each represents a different hemocyte 'type'.

The cladogram constructed from the data matrix of different hemocytes from Table 3 is shown in Figure 12.

FIGURE 11

Morphologically distinct hemocytes seen in hemolymph smears of Psychoglypha alascensis. Cells have been numbered to facilitate further reference. (Giemsa, 2200x).



11

Table 3. Data matrix for cells 1-24 of Psychoglypha alascensis (Figure 11). All measurements in mm.^{2} from graph paper tracings of cells in Figure 11. Definitions of character states are given below.

A- Area of cell (nuclear area + cytoplasmic area)

0= 10
1= 12.5 to 15.5
2= 21 to 27
3= 32

B- Area of nucleus

0= 6 to 11.5
1= 15 to 19

C- Cytoplasm/nucleus area

0= nucleus area greater than cytoplasm area
1= 1.1:1 to 1.28:1
2= 1.56:1 to 1.9:1
3= 3.3:1 to 3.9:1
4= 4.3:1

D- Nucleus position

0= central
1= eccentric

E- Shape of cell

0= round
1= some variation of spindled
2= presence of pseudopodia

F- Compactness of nucleus

0= dense
1= loose

G- Compactness of cytoplasm

0= dense
1= loose

H- Cytoplasm granulation

0= no granulation
1= some small granules

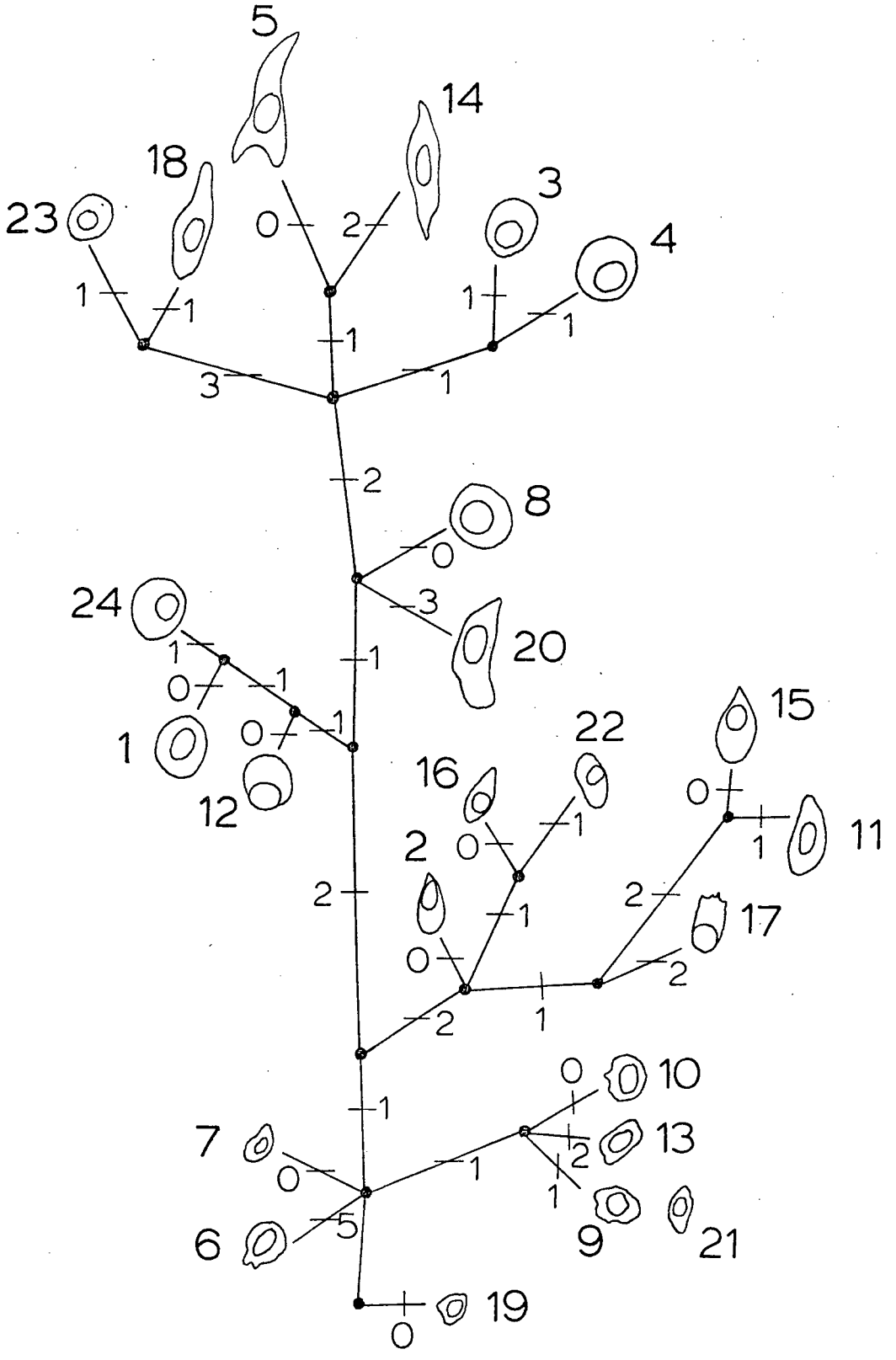
I- Cytoplasm vacuolation

0= no vacuolation
1= vacuolation

		Cell Character								
		A	B	C	D	E	F	G	H	I
Cell Number	1.	2	0	3	1	0	1	0	0	0
	2.	1	0	2	1	1	0	0	0	0
	3.	2	1	2	1	0	1	0	0	1
	4.	3	1	2	1	0	1	1	0	1
	5.	2	1	2	0	1	1	1	0	1
	6.	0	0	0	1	2	0	0	0	0
	7.	1	0	1	0	0	0	0	0	0
	8.	2	1	2	0	0	1	0	0	0
	9.	1	0	0	0	0	0	1	0	0
	10.	1	0	1	0	0	0	1	0	0
	11.	2	0	2	0	1	1	0	0	1
	12.	2	0	2	1	0	1	0	0	0
	13.	1	0	1	0	2	0	1	0	0
	14.	2	1	2	0	1	0	1	0	0
	15.	2	0	2	1	1	1	0	0	1
	16.	1	0	2	1	1	0	0	0	1
	17.	2	0	2	1	2	0	1	0	0
	18.	2	0	3	0	1	0	1	0	1
	19.	0	0	0	0	0	0	0	0	0
	20.	2	1	3	0	1	1	1	0	0
	21.	1	0	0	0	0	0	1	0	0
	22.	1	0	2	1	1	0	1	0	1
	23.	2	0	3	1	0	0	1	0	1
	24.	2	0	4	1	0	1	0	0	0

FIGURE 12

Cladogram of hemocytes of Psychoglypha alascensis. Constructed from data matrix in Table 3. Numbered hemocytes correspond to those in Figure 11. The number of synapomorphies characterizing each branch is indicated at each mid-branch point.


5
14
18
3
23
4
8
24
20
1
12
22
16
15
2
11
17
10
7
13
6
9
21
19

The particular unique characters (synapomorphies) are marked in along each branch. When the branches not characterized by any synapomorphies (with zero branch lengths) are collapsed down to the nodes, the tree becomes a Summary Fate Map of possible developmental pathways open to developing hemocytes (Caira, Brooks, Scudder, in prep.) (Figure 13). The Summary Fate Map postulates six hemocyte types of P. alascensis which were not seen on the slides examined for hemocytes. These unseen types are represented by black circles on the fate map in Figure 13.

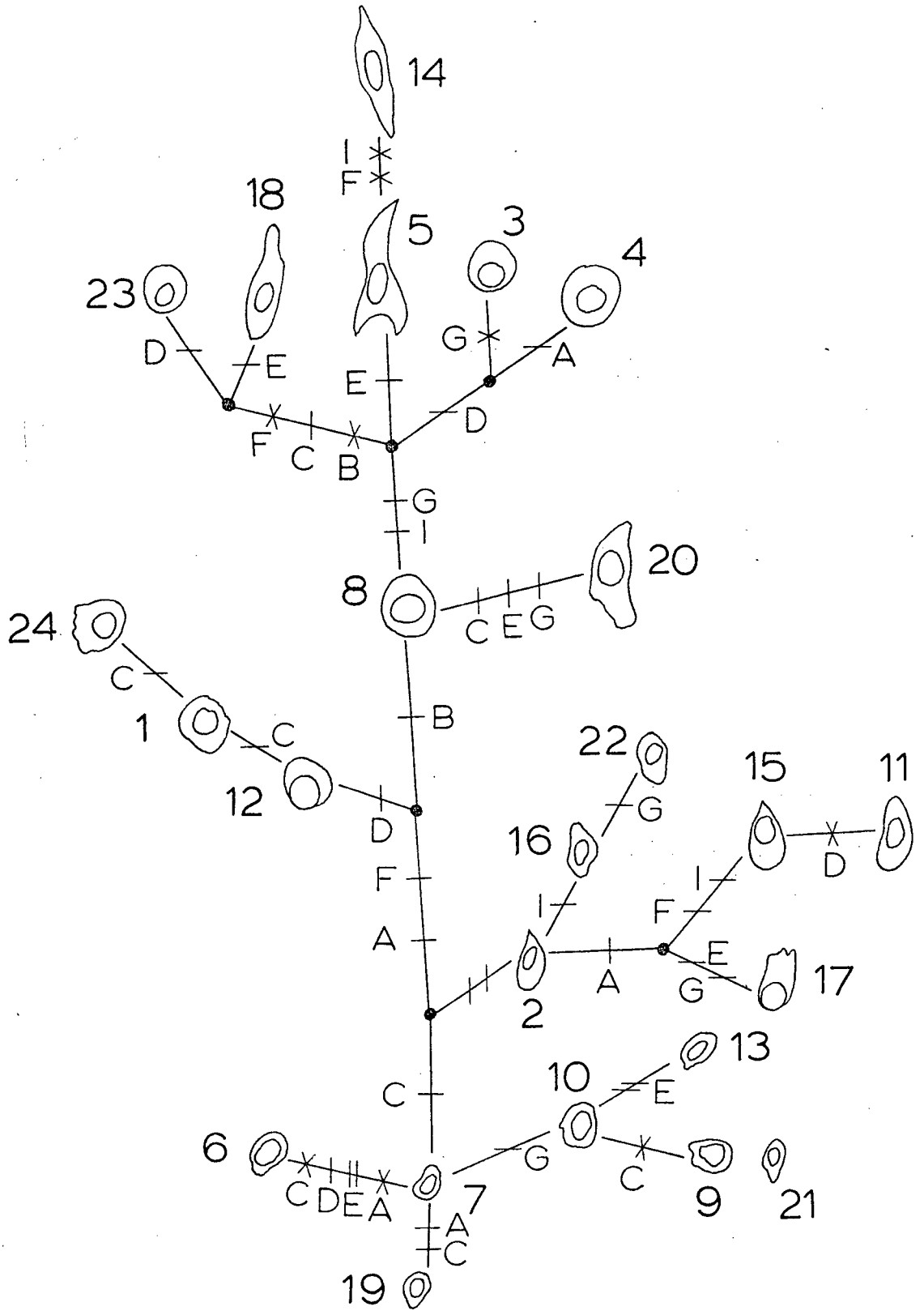
In summary then, examination of hemocytes revealed at least 24 morphologically distinct types of hemocytes. Cladistic analysis of these cells showed that many of the hemocytes seen were stages in the development of several lines of distinct hemocyte types.

H. Reaction to Implants

The silk glands are an unusual site in which to find digenean encystment and it is not a site in which one would expect to find a hemocyte reaction to a parasite. The gland lies within the hemocoele, however, the lumen of this gland is not part of the hemocoele, and consequently does not possess a normal hemocyte population. Before examining the response of P. alascensis to a parasite encysted within the silk gland, it seemed advisable to examine the hemocyte response of P. alascensis to foreign

FIGURE 13

Summary Fate Map of hemocyte lineages. Produced by collapsing all cells of Psychoglypha alascensis with zero branch lengths in the cladogram in Figure 12 to nodes. Synapomorphic characters (corresponding to those in Table 3) are marked by letter along each branch. Numbered hemocytes correspond to those in Figure 11. /= a single forward shift in a character state, //= two consecutive forward shifts in a character state, X= a reversal of one state of the character, ●= precursor cell (node) postulated by Wagner analysis of the data matrix (Table 3), but not found among cells examined in hemolymph smears.


14
I
F
18
5
3
4
23
D
E
G
A
E
D
F
C
B
G
I
8
20
C E G
24
C
1
C
12
B
22
G
15
11
D
16
D
F
I
F
I
A
E
A
G
17
2
13
C
10
E
6
G
C
9
21
C D E A
7
A
C
19

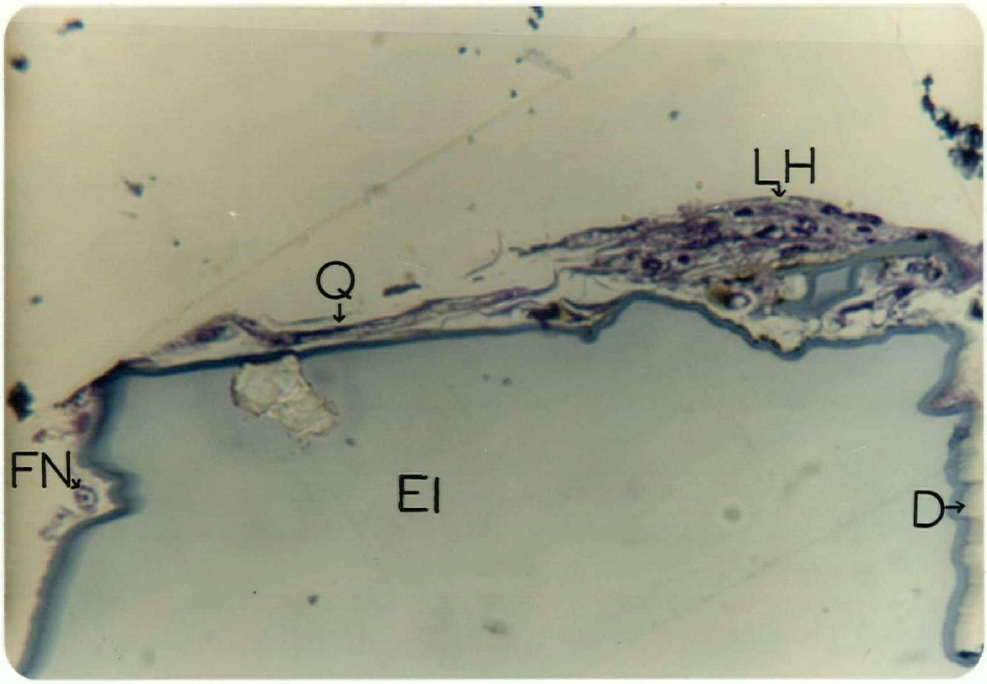
objects within an area accessible to hemocytes such as the hemocoele. Hence the competence of the cellular immune system of P. alascensis was tested by examining the response of the larvae to inert pieces of Epon implanted within the hemocoele. Two response periods were chosen, one short (approximately 7 days), and one long (approximately 35 days).

(i) Seven Day Implants

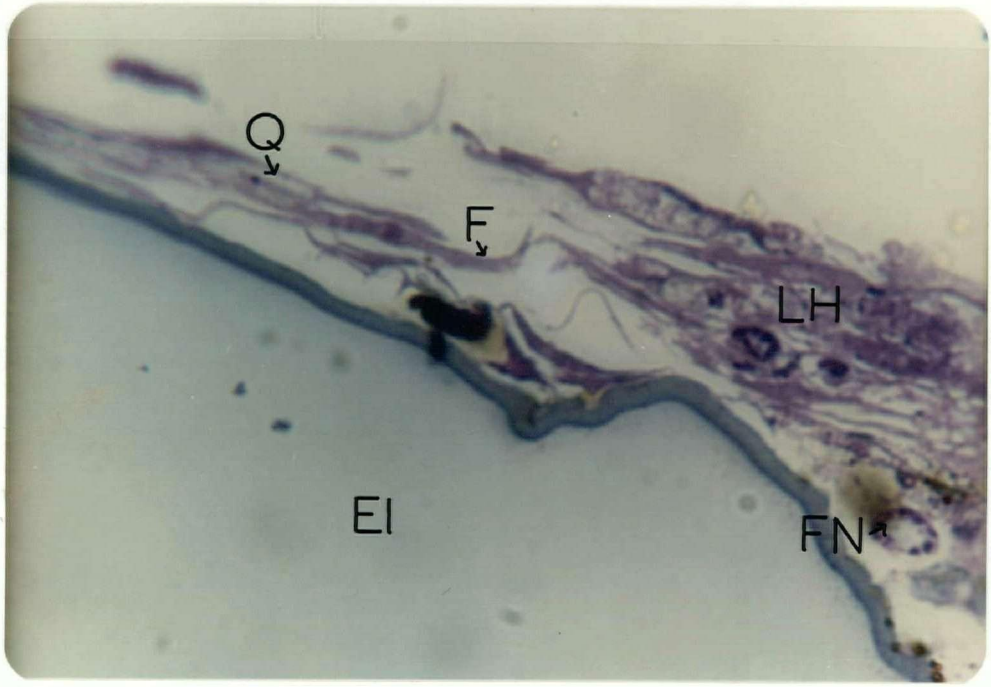
Of the six larvae receiving implants, one died before the seven day period was complete. In another larva, one of the implants had moved into the area of the incision so that one-half of the Epon was within the animal while the other half was exposed to the air. As part of the reaction around this implant was the closing of the integumental wound, it was not examined for an encapsulation reaction. Owing to the small size and transparent nature of the implants, dissection of larvae receiving implants led to the retrieval of Epon slivers from only two of the four remaining individuals. Sections of these slivers showed a spotty hemocyte response. A section of one of these Epon slivers is shown in Figure 14. Some areas of the implant appear completely free of hemocytes (D in Figures 14 a and b). In other areas, however, several hemocytes have become greatly extended and have flattened against the implant, following the contours of its edge (Q in Figures 14a and b). In yet other areas of the implant groups of hemocytes have flattened

FIGURE 14

Cross sections of the hemocyte response of Psychoglypha alascensis to an Epon sliver implanted in the abdominal hemocoele and removed after seven days, (a) (Methylene blue, 675x), (b) (Methylene blue, 2200x). D= hemocyte-free area of implant, EI= Epon implant, F= flattened hemocyte, FN= free hemocyte nucleus, LH= area of implant covered by capsular layers of hemocytes, Q= area of implant covered by only a few hemocytes.



a



b

against the edge of the implant forming part of a capsule of hemocytes approximately 15-20u in diameter (L in Figures 14a and b). Several hemocytes had nuclei which stained distinctly with large peripheral chromatin granules, as well as several central and eccentric chromatin granules. In some areas only nuclei of hemocytes are visible directly against the surface of the implant (FN in Figure 14a and b). This, however, does not appear to be the case in all areas of the implant; in some areas flattened but intact hemocytes are visible (FL in Figure 14b) directly against the surface of the implant.

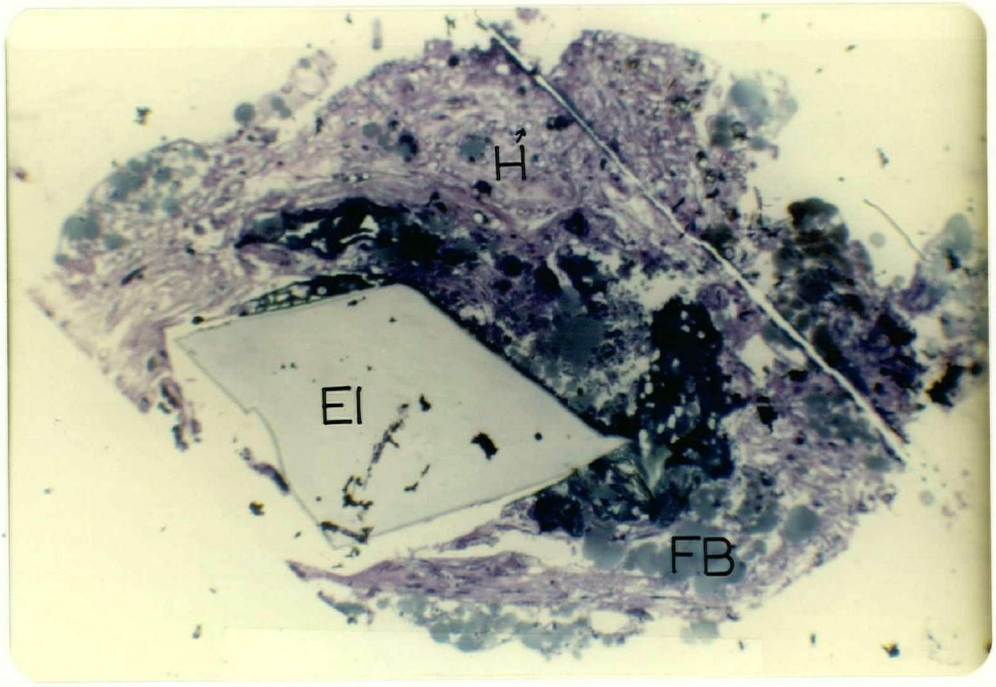
(ii) Thirty-Five Day Implants

Four of the six larvae receiving Epon implants were still alive 35 days later; dissection led to the retrieval of Epon slivers from three of the four remaining individuals. Again, however, one of these implants was located directly within the wound healing reaction of the integument and thus not examined. Sections of the Epon slivers showed them to be almost totally surrounded by hemocytes (Figure 15). Included in the hemocyte capsule was some darkly staining fat body tissue (FB in Figure 15).

It appeared that after 35 days the hemocyte capsule around the implant and fat body tissue was composed of two distinct parts, an inner area of densely packed hemocytes (30-70u in width), and an outer area of loosely packed hemocytes (30-80u in width). In the inner dense area of the capsule, both the hemocytes and their

FIGURE 15

Cross section of the response of Psychoglypha alascensis to an Epon sliver implanted in the abdominal hemocoele and removed after 35 days. EI= Epon implant, FB= fat body tissue, H= hemocyte (Methylene blue, 200x).



15

nuclei were extremely flattened and the cellular and nuclear boundaries of the hemocytes were very difficult to discern (I in Figure 16). In most areas the hemocytes adhered directly to each other with little or no intercellular spacing.

In contrast to the cells of the inner layer, the hemocytes of the outer area of the capsule were only slightly flattened (O in Figure 16). In some areas round nuclei with peripheral and eccentric chromatin granules were clearly visible. In addition, there appeared to be more intercellular space than in the inner layer, and in some areas hemocyte boundaries were clearly visible.

It appears then, that the hemocytes of P. alascensis are capable of responding to foreign objects located in the hemocoele after seven days and of totally encapsulating them after 35 days.

I. Host Response to the Parasite

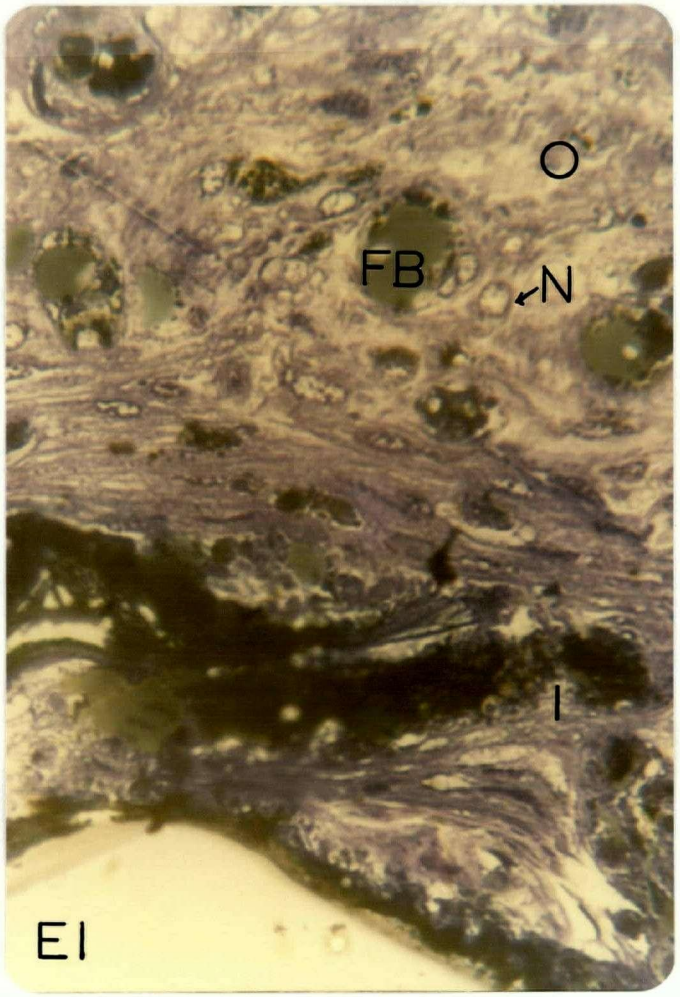
Parasite infections were judged to be "new", "well established" or "old" on comparative morphology and are described separately below.

(i) New Infections

In silk glands removed from field collected caddisfly larvae, several metacercariae did not appear to be situated directly within the lumen of the gland. When these glands were sectioned, it was discovered that the metacercariae were actually situated within the epithelial layer of the silk

FIGURE 16

Magnified view of response of Psychoglypha alascensis to a 35 day old Epon implant. EI= Epon implant, FB= fat body tissue, I= inner layer of capsule, N= hemocyte nucleus, O= outer layer of capsule. (Methylene blue, 675x).



16

gland wall. These metacercariae were small (100-105u in diameter) and appeared to have a thin cyst wall. I therefore, considered these metacercariae to be immature, however, their exact ages were not known. One of these young metacercariae is shown in Figure 17. The epithelial layer of the silk gland both adjacent to the metacercaria for about 100u and in the wall of the gland opposite the metacercaria for about 200u, appeared to be degenerating (DE in Figure 17). The epithelium was discontinuous and much thinner than the normal epithelium of the silk gland (5-10u as opposed to 10-20u). In addition, hemocytes appeared to have moved to some areas of the degenerating epithelial layer both adjacent to and across from the penetrating metacercariae.

Figure 18 is a magnified view of the area of the gland adjacent to the metacercaria. The hemocytes in this area appeared flattened in some sections, but retained their rounded outline in others.

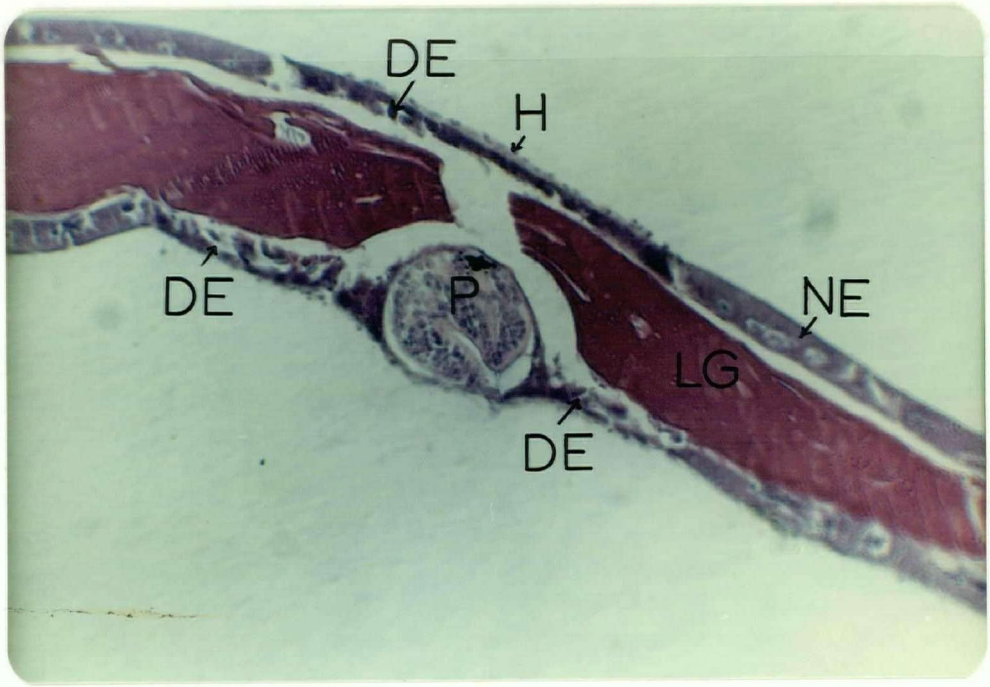
Figure 19 shows the area of the gland opposite the penetrating metacercaria. The flattened nuclei and cytoplasm of the hemocytes are readily distinguishable. In most areas two to three layers of hemocytes are present. The hemocytes lying directly against the epithelium of the silk gland are very flattened, the nuclei are 1-2u in height. Hemocytes composing the second and third layer of the capsule are not as flattened, the nuclei are 2-4u in height.

FIGURE 17

Longitudinal section of a young metacercaria of Bunodera mediovitellata within the epithelial wall of the silk gland of Psychoglypha alascensis. DE= degenerated epithelium, H= hemocyte, LG= lumen of the silk gland, NE= epithelial cell nucleus, P= parasite. (Hematoxylin and eosin Y, 200x).

FIGURE 18

Magnified view of a longitudinal section of the silk gland epithelium adjacent to the parasite. DE= degenerated epithelium, H= hemocyte, LG= lumen of the silk gland, P= parasite. (Hematoxylin and eosin Y, 675x)



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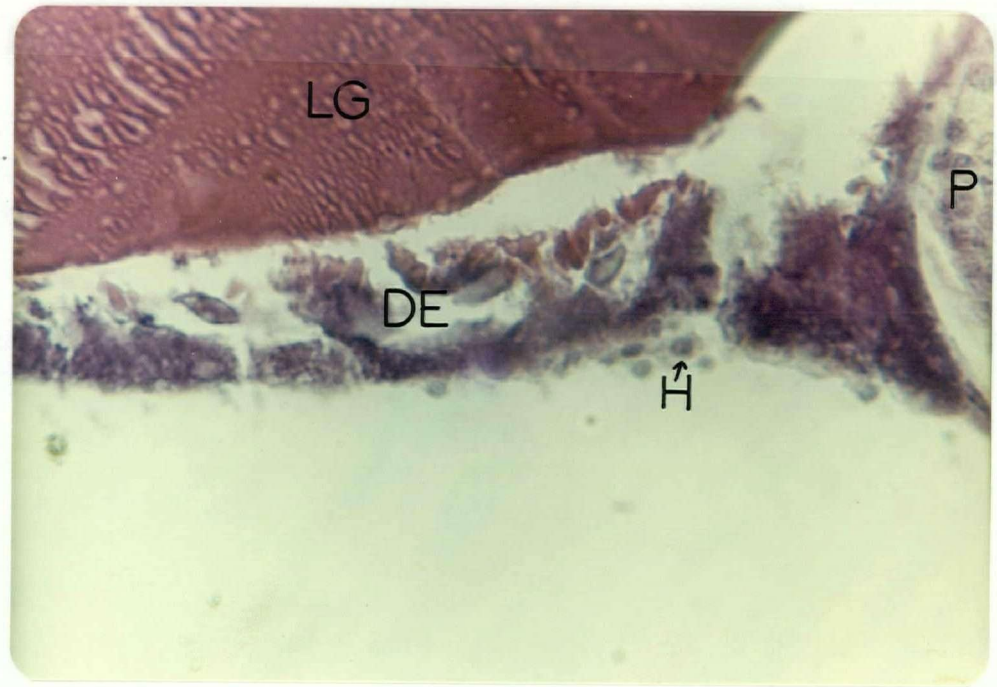

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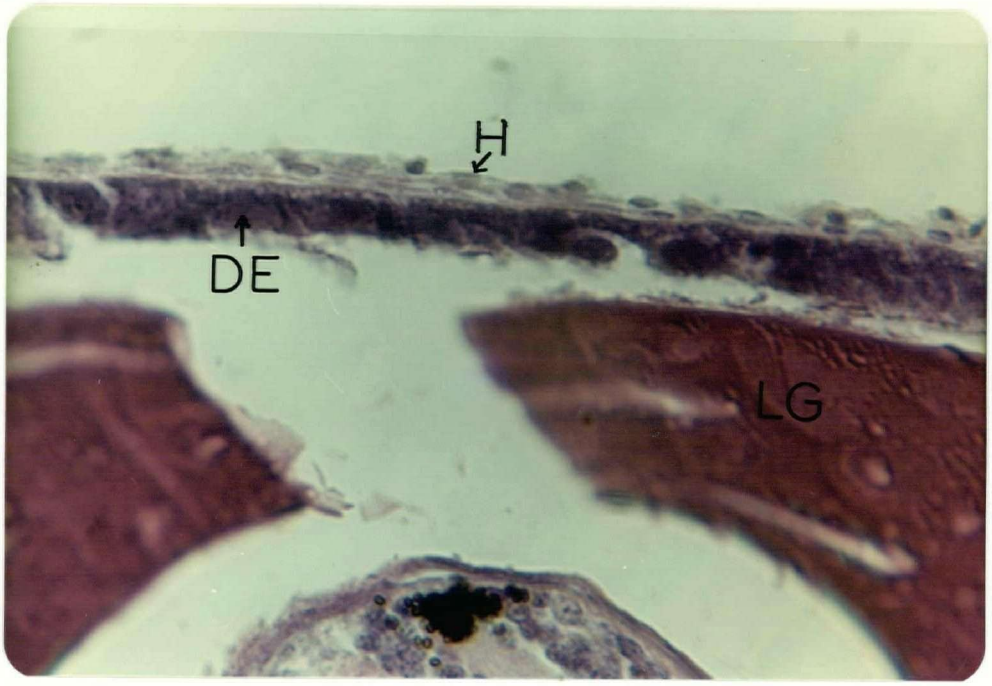
FIGURE 19

Magnified view of a longitudinal section of the silk gland epithelium across from the parasite. Demonstrating flattened hemocytes against the surface of the silk gland. DE= degenerated epithelium, H= hemocyte, LG= lumen of the silk gland. (Hematoxylin and eosin Y, 2200x).



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Therefore, it seems that before the metacercariae actually contacted the opposite wall of the silk gland, the epithelium of the gland had begun to degenerate and hemocytes had moved to the walls of the silk gland.

(ii) Well Established Infections

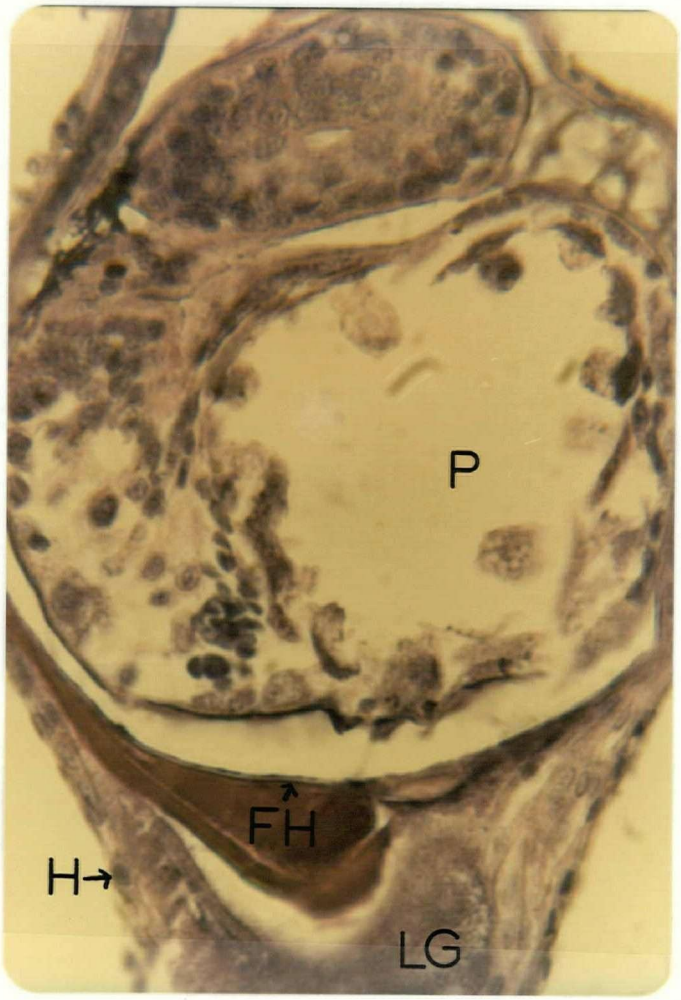
The majority of metacercariae taken from field collected caddisfly larvae were situated directly within the lumen of the posterior one-third of the silk glands. Sections showed the cysts to totally occlude the lumen of the silk gland, as the cysts were wider than the gland itself (130-190u) (Figure 20). Consequently, silk produced by the area of the gland lying between the metacercaria and the blind end of the gland could no longer move forward. This distal area of the gland usually showed signs of degeneration.

Light microscope sections of Epon embedded metacercariae stained with methylene blue showed the cyst wall secreted by the parasite as an amorphous purple-pink layer lying directly adjacent to the parasite (C in Figure 21). Next to the cyst wall in some sections was a thin layer of silk which appeared to be amorphous and stained blue (S in Figure 21). In some cases this layer of silk was not present, probably owing to the growth of the parasite pushing the silk away from this area.

The area next to the silk was a densely layered area shich stained a very dark blue (I in Figure 21). Hemocyte nuclei were visible in some areas of this

FIGURE 20

Longitudinal section of a metacercaria of Bunodera mediovitellata established within the silk gland of Psychoglypha alascensis. FH= area of parasite free of hemocytes, H= hemocyte, LG= lumen of the silk gland, P= parasite. (Hematoxylin and eosin Y, 675x).



20

FIGURE 21

Magnified views of two areas of a longitudinal section through a metacercaria of Bunodera mediovitellata encysted within the silk gland of Psychoglypha alascensis, (a) (Methylene blue, 2200x), (b) (Methylene blue, 2200 x). A= amorphous pink substance, C= cyst wall, F= flattened hemocyte, I= inner layer of the capsule, N= hemocyte nucleus, O= outer layer of the capsule, R= rounded hemocyte, S= silk.

b

a

layer (N in Figure 21). However, the majority of the hemocytes were so densely packed that the cell outlines were not clear and the layer appeared fibrous. There was no evidence of the epithelial layer of the wall of the silk gland in this area. In addition, amorphous masses of homogeneous material which stained pink with methylene blue were visible at the inner area of this layer (A in Figure 21).

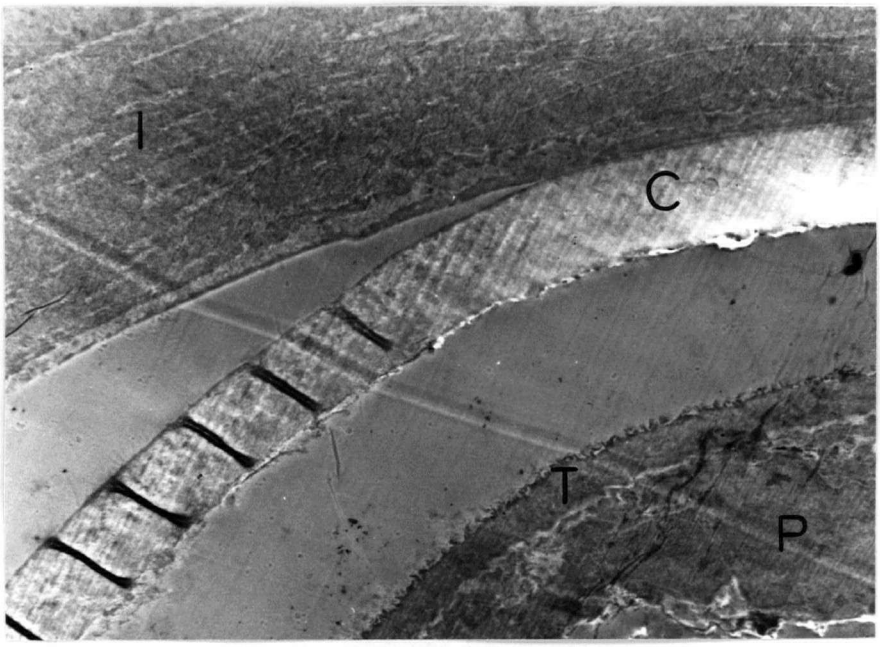
The outermost layer around the metacercaria was one of loosely packed hemocytes which stained very light blue (O in Figure 21). In many areas individual cells were easily recognizable. The hemocytes in this outer layer were not as flat as were those in the inner layer and there was much intercellular space. In several areas unflattened hemocytes were visible towards the outer area of the capsule (R in Figure 21). In this outer area there was no evidence of the epithelium of the silk gland.

It appeared that the wall of the silk gland around the parasite had been totally replaced by a capsule composed of two layers of hemocytes, one containing densely packed cells and one containing loosely packed cells.

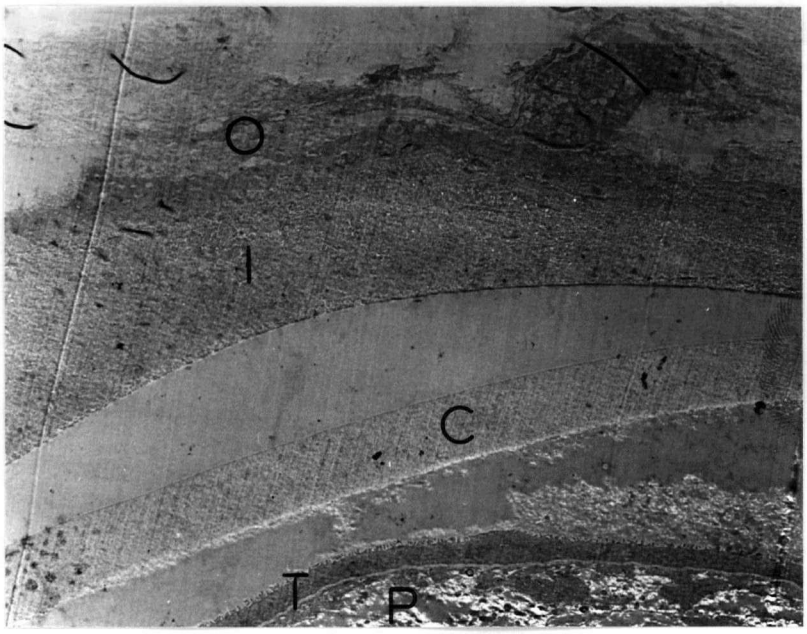
Electron micrographs of sections through metacercariae encysted within the lumen of silk glands, support the above observations. Visible against the tegument of the parasite is the amorphous cyst wall approximately 5u in thickness (C in Figures 22a and b). Next to that there appeared to be a fibrous layer composed of compact cells (I in Figures 22a

FIGURE 22

Electron micrographs of capsules around metacercariae of Bunodera mediovitellata encysted within the lumen of a silk gland of Psychoglypha alascensis, (a) demonstrates inner layer of the capsule (2500x), (b) demonstrates outer layer of the capsule (1875x). C= cyst wall, I = inner layer of capsule, O= outer layer of capsule, P= parasite, T= tegument of parasite.



a



b

and b); details of the cells were not visible. The electron micrographs do not show the details of the outer layer of the capsule, although loosely 'packed' material was visible at the outside edge of the capsule in one section (O in Figure 22b).

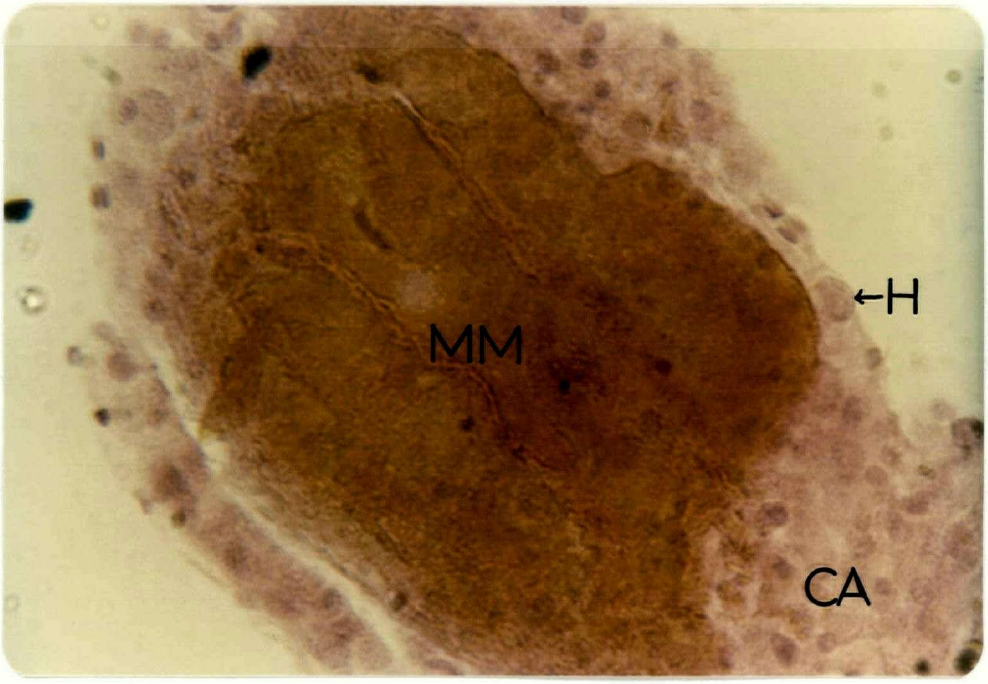
It appears that hemocytes do not immediately enter into the lumen of the silk gland and totally encapsulate the metacercaria upon its penetration. Sections of some metacercariae clearly showed that the hemocytes had totally replaced the epithelial wall of the silk gland, both directly around the parasite, and approximately 150u on either side of the parasite, so that the capsule was surrounding both the parasite and the silk of the gland. However, longitudinal sections of these capsules showed that the hemocytes had not passed through the silk to associate with the areas of the parasite remote from the gland wall (FN in Figure 20). Therefore, areas of the parasite remained free of hemocytes within the lumen of the gland.

In sections of other capsules, probably of older infections, hemocytes had moved in so that all areas of the parasite were encapsulated. In these sections, hemocytes had blocked the lumen of the gland on either side of the parasite so that the metacercariae were isolated from the rest of the gland (Figure 23).

Several of the silk glands sectioned contained more than one metacercaria within the lumen of the gland.

FIGURE 23

Transverse section of a melanized metacercaria of Bunodera mediovitellata in which hemocytes have entered the silk gland lumen and attached to the parasite. All of the surface of the parasite is covered with hemocytes. CA= area in which hemocytes have entered the lumen of the silk gland and attached to the parasite, MM= melanized metacercaria. (Hematoxylin and eosin Y, 675x).



23

In multiple infections such as this, all metacercariae were surrounded by flattened hemocytes (Figure 24). In addition, the epithelium of the silk gland lying between each metacercaria had often been replaced by hemocytes (H in Figure 24); in no case was epithelial regeneration detected.

Metacercariae inside many of these cysts appeared to be intact. When several of the unfixed cysts were broken open, the metacercariae released were alive and able to move.

(iii) Old Infections

Metacercariae removed from a total of 15 larvae of P. alascensis collected in late July and August of both 1979 and 1980 were found to be covered with a brown pigment. In wax sections of metacercariae stained with hematoxylin and eosin Y this material appeared as a brittle amorphous brown substance lying between the inner layer of the hemocyte capsule and the cyst wall of the parasite (Figure 25).

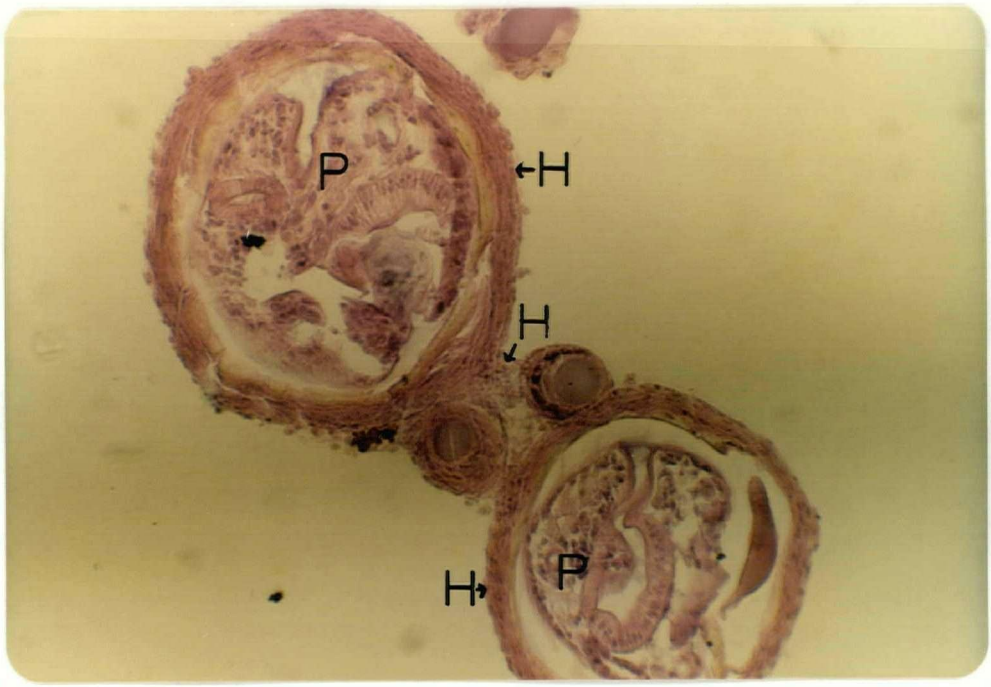
When these brown metacercariae were sectioned and treated with the Ferrous Iron Technique for melanin (Pearse, 1972), the brown areas stained a dark greenish brown, while the metacercariae and the rest of the silk gland stained light green. When other sections of these metacercariae were treated with potassium permanganate and oxalic acid, a melanin specific bleach followed by the melanin specific stain, all areas of the capsule (including what was

FIGURE 24

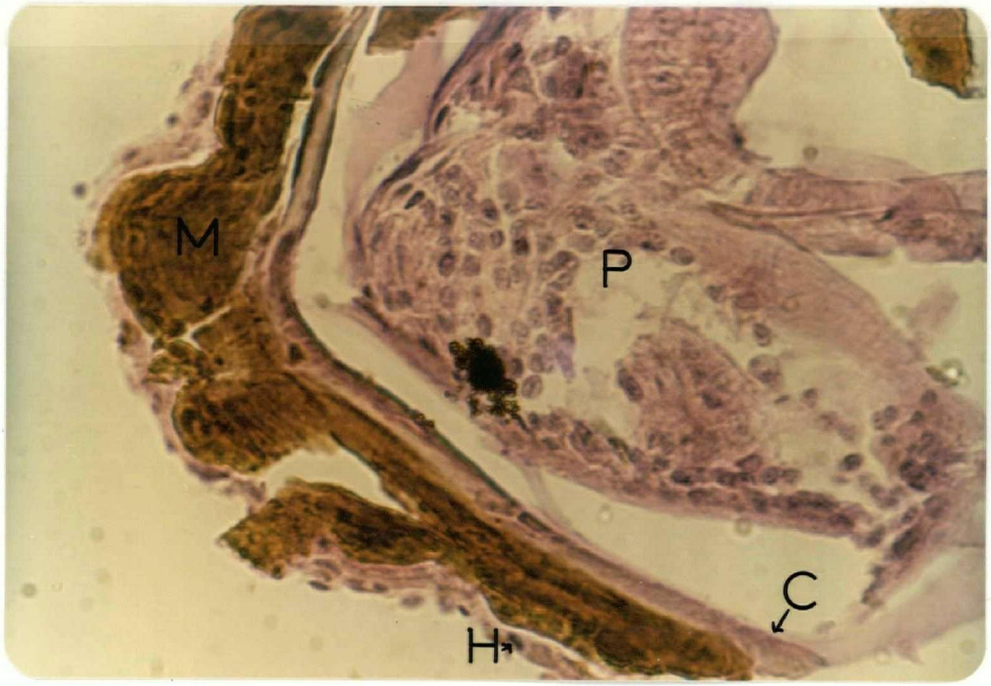
Cross section of a silk gland of Psychoglypha alascensis containing two metacercariae of Bunodera mediovitellata. Note that the silk gland epithelium between the two metacercariae has been replaced by hemocytes. H= hemocyte, P= parasite. (Hematoxylin and eosin Y, 200x).

FIGURE 25

Longitudinal section through a melanized metacercarial cyst of Bunodera mediovitellata. C= cyst wall, H= hemocyte, M= melanin, P= parasite. (Hematoxylin and eosin Y, 675x).



24



25

originally brown pigmentation) as well as the metacercaria and silk gland stained light green. Therefore, the brown pigmentation was considered to be melanin.

In all of the melanized metacercariae sectioned, the lumen of the silk gland next to the parasite had been totally blocked by hemocytes. This finding and the fact that melanized metacercariae were consistently found only at the end of the summers, suggests that the melanization of the parasite occurred only after the parasite had been within the caddisfly larva for a long period of time, perhaps two or three months. It may be noted that even after this period of time, the hemocytes replaced the silk gland epithelium but did not form a massive unorganized capsule. Instead, they formed a structure very similar in diameter and shape to the original silk gland epithelium.

The metacercariae within the melanized silk glands usually did not appear intact (P in Figure 25). However, it is difficult to determine whether or not this is an artifact of sectioning owing to the tough melanin.

J. Hemocytes Involved in the Encapsulation Response

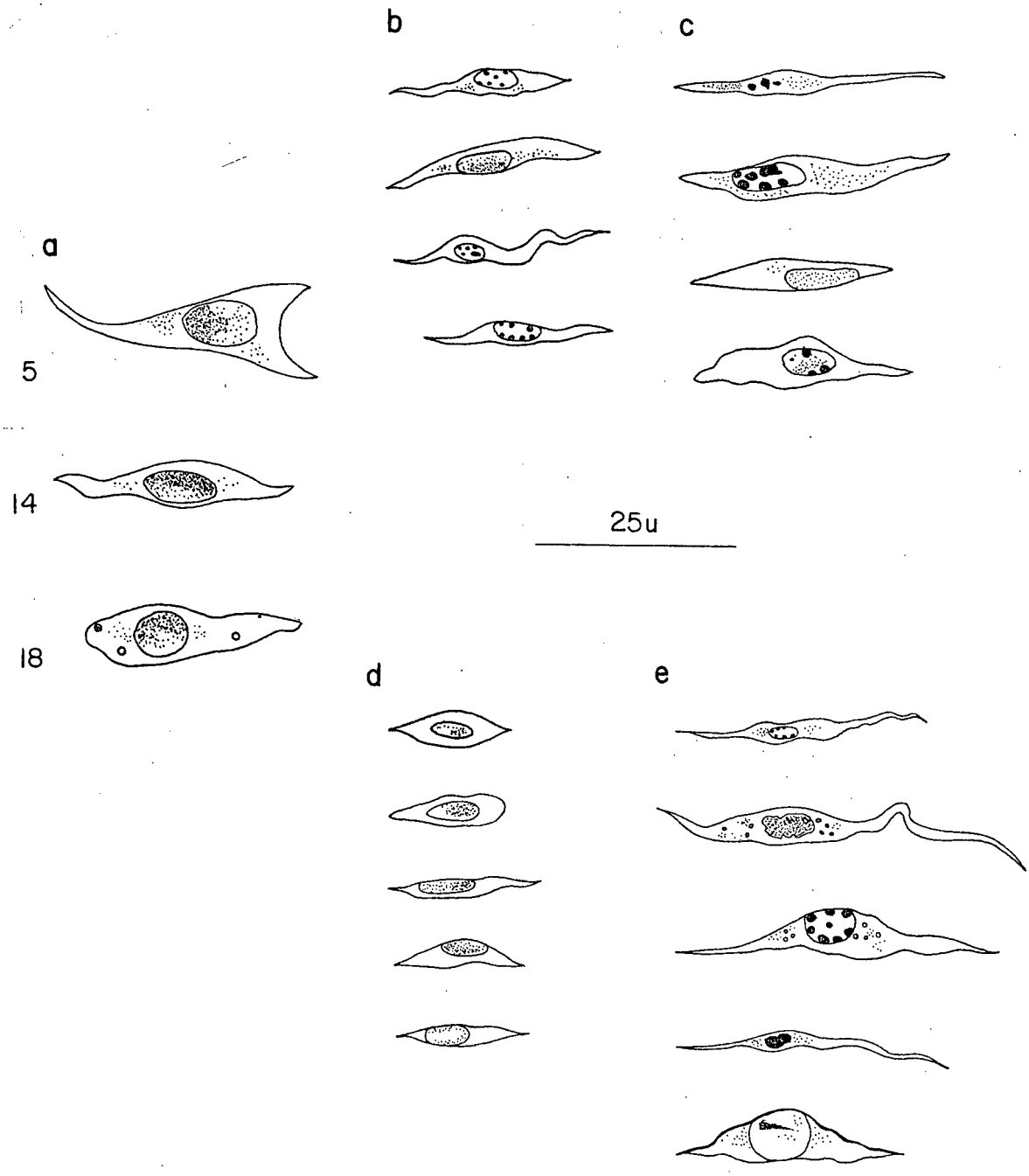
In both the 35 day capsule formed around the Epon implants and the established metacercarial capsule, the cytoplasm and nuclei of the hemocytes forming the dense inner layer of the capsule had become so flattened and extended that the extent of the cytoplasm and nuclei were difficult to determine and consequently particular types of hemocytes

were not recognizable. However, the hemocytes forming the outer layer of the older metacercarial capsules, the outer part of the 35 day implant capsule, as well as those taking part in the response to the seven day implant, and the new metacercariae were not as flattened and sections of whole hemocytes were often visible.

To facilitate the comparison between the freely circulating hemocytes and cell types taking part in the encapsulation response, a sample of the longest cells seen in each of the reactions to the seven day implants, 35 day implants, young metacercariae, and established metacercariae was taken and sketched to scale with the aid of a drawing tube (Figure 26). These were then compared with the types of hemocytes seen in hemolymph smears (Figure 11). It should be stressed that while the sketches of free circulating hemocytes are of whole cells, the sketches of the capsule cells are of cross sections. Because of the complications that may be involved with cutting cross sections of non-symmetrical cells at various angles, I chose to sample only the longest cells seen in the above capsules; in interpreting sections showing small cells, I had no way of distinguishing between sections cut across the axis of long thin cells (therefore, appearing as small cells) and sections cut longitudinally through truly small cells. However, two of the melanized metacercariae were cut sagitally rather than transversely and hence show many of the hemocytes involved in the capsules.

FIGURE 26

Drawings of sample hemocytes from hemolymph smears and involved in outer layer of capsules. Drawings done with aid of a drawing tube. (a) Free circulating hemocytes from hemolymph smears; numbers correspond to those in Figure 11. (b) Hemocytes from cpasule around seven day Epon implants. (c) Hemocytes from capsule around 35 day Epon implants. (d) Hemocytes from capsule around new metacercariae. (e) Hemocytes from capsules around established metacercariae.


a
b
c
5
14
18
25u
d
e

Unfortunately the limits of the cells seen in the sagital sections were not visible.

The sections of hemocytes taking part in the capsule around the seven day implants are shown in Figure 26b. These cells appear to be longer than those taking part in the capsule around the new metacercariae (Figure 26a). These cells are as long as cell #14 and cell #18, however, no cells seen were as long as cell #5. As the preparations are of cross sections of cells, characters such as cytoplasm area, nucleus area, and cytoplasm and nucleus density cannot be used, and subsequently a more detailed comparison with the free-circulating cells cannot be made.

A sample of the large hemocytes taking part in the response to the new metacercariae (Figure 26d) show cells that are smaller than any of the three longest free-circulating cell types, and correspond more closely to the size of cell #8 and #3 from the free-circulating hemocyte smears. Again as these cells are cross sections no further correlation between these and the free-circulating cell types can be made.

Figure 26c shows the large cells seen taking part in the reaction to the 35 day implant. These cells are long enough to correspond to free-circulating cells #14, 18, and 5. Several of the cells appear to have distinctly eccentric nuclei and therefore would correspond most closely to #18.

The cells in Figure 26e are those seen taking part in

the response to well established metacercariae. Three of these cells are longer than the longest free-circulating cell type seen(#5). It will be noted that the edges of these cells are extremely thin (c.f. those around the new metacercariae), perhaps indicating elongation of the cells as encapsulation progresses. From this study however, I am unable to determine whether these cells represent new cells or elongated versions of the free-circulating cell types seen.

Only the nuclei of hemocytes taking part in the melanized reactions were visible; the cytoplasmic boundaries of the cells were not visible. These nuclei were of equal density and had a size range from 4 to 9u. Without the cytoplasmic boundaries of the cells, correlations between these and the free-circulating hemocyte types could not be made.

In summary then, it appears that a number of different hemocytes may respond to the metacercariae located within the silk glands. In particular it is suggested that cells similar to free-circulating cells, # 14, 18, 5, and several cells larger than #5 may participate in the reaction. Those that do respond may flatten and extend their cytoplasm as the encapsulation proceeds. This would account for the extremely flattened cells in the inner layer of older capsules.

IV. DISCUSSION

A. Parasites of Trichoptera and the Incidence of the Parasite Bunodera mediovitellata in its Trichopteran Host

Of the five major groups of metazoan parasites, Insecta, Digenea, Cestoda,Nematoda and Acanthocephala, Salt (1963) suggests that digeneans are the only group that parasitize the Trichoptera. To my knowledge this statement remains true to date. However, not all digeneans are parasites of caddisflies. In fact, of the thirteen families of Digenea other than the allocreadiids, known to parasitize aquatic insects, (Hall, personal communication) only three have been reported from Trichoptera. These include members of the Lecithodendriidae, Gorgoderidae, and Plagiorchiidae. The species within each family recorded from Trichoptera are given in Table 4.

There was a large difference in the incidence of parasitism by B. mediovitellata between the two species of Trichoptera. Since (1) cercariae were seen to penetrate both species of caddisfly, and (2) there appeared to be no difference between the cysts of B. mediovitellata within the silk glands of L. roafi and those within P. alascensis, it appears that once the cercariae are in the vicinity of either species of caddisfly larva they will penetrate and encyst. However, the fact that fewer metacercariae were found naturally infecting larvae of L. roafi than larvae of P. alascensis suggests that in general, larvae of L. roafi were not in the vicinity of

Table 4. Families of Digenea, excluding the Allocreadiidae, whose members parasitize Trichoptera. G= Gorgoderidae, L= Lecithodendriidae, P= Plagiorchiidae, NM= Information not mentioned in reference.

Species of Digenean	Larval Stage	Trichopteran Host	Site in Host	Reference
(L) *Acanthatrium oregonense* Macy	metacercariae unencysted	*Limnephilus* sp. (larvae)	fat body	Knight and Pratt (1955)
	metacercariae unencysted	*Dicosmoecus* sp. (larvae)	abdominal hemocoel, gills	Burns (1961)
(L) *Allasogonoporus vespertillionis* Macy	metacercariae encysted	*Limnephilus* sp. (larvae)	fat body	Knight and Pratt (1955)
(L) *Lecithodendrium chilostomii* (Mehl.)	metacercariae 1° unencysted	*Phryganea grandis* L. (larvae)	free within body cavity, fat body	Brown (1933)
	metacercariae 2° encysted	*Phyrganea grandis* (pupae and adults)	thoracic hemocoele	Brown (1933)
(L) *Pleurogenes medians* (Olss.)	metacercariae encysted	*Limephilus* sp. (larvae) *Sericostoma* sp. (larvae)	connective tissue of gill, body cavity nearby	Neuhaus (1940)
(G) *Phyllodistomum staffordi* Pearse	metacercariae unencysted	Trichoptera (larvae)	thoracic	Schell (1967)
(P) *Distomum endolobum*	metacercariae encysted	*Limnephilus lunatus* Curtis, *L. griseus* (L.), *L. rhombicus* (L.), *Anabolia nervosa* Leach	NM	Linstow (1897)

Table 4. (continued)

(P) Distomum isoporum	metacercariae encysted	Chaetopteryx villosa F., Anabolia nervosa	NM	Linstow (1897)
(P) Distomum mystacidis	metacercariae encysted	Mystacides nigra (L.) Limnephilus flavicornis (Fab.)	NM	Linstow (1897)
(P) Opisthoglyphe locellus Kossack	metacercariae encysted	Trichoptera (larvae)	NM	Macy and Moore (1958)
(P) Plagiorchis goodmani Najarian	metacercariae encysted	Limnephilus indivisus Walker (larvae)	NM	Najarian (1952)

the cercariae at the time of their release, whereas, the larvae of P. alascensis were. Unfortunately I did not have time to carry out a detailed study of the natural habits of each species of caddisfly. However, I can speculate as to three circumstances which may account for L. roafi having less contact with B. mediovitellata.

Firstly, it is possible that the two species of caddisfly normally inhabit areas at different depths in the stream. There is indirect evidence to suggest that this is true. When buckets containing caddisfly larvae collected from the field were examined, larvae of L. roafi were consistently found attached to the walls of the buckets just below the surface of the water. Larvae of P. alascensis rarely attached to the walls of the buckets; they were usually located on the floor of the buckets along with the clams.

Secondly, it is possible that the life cycles of L. roafi and P. alascensis differ such that during the time when cercariae are released from clams, larvae of L. roafi are too small to be infected, or are less abundant than those of P. alascensis. Several times over the 24 month collection period no larvae of L. roafi were found. In contrast larvae of P. alascensis were always found, although at times in small numbers.

Lastly, it is possible that owing to the larger size of the case, the larvæ of P. alascensis have a stronger ventillation force and thus are able to pull more

cercariae into their vicinity. However, if this were the case one would expect that animals with large cases would be infected with more than one metacercariae more often than those with small cases. The analysis of variance demonstrated however, that the size of the case of the individual did not appear to affect the number of cysts per individual.

The incidence of parasitism appeared to be seasonal. In both larvae, there was a low incidence of parasitism at the beginning of spring, but this then increased to a high incidence in July of both sampling years. These seasonal differences may reflect the anadramous nature of the stickleback definitive host. According to Kennedy (personal communication) sticklebacks move up the stream from its outlet at the ocean at various times during the year. It is possible that the infected sticklebacks move up the stream in May. At this point eggs from B. mediovitellata are released with the feces of the fish and thus the cycle of the parasite begins so that by July cercariae have been released from the clams and have infected the caddisflies. In support of this, the analysis of variance indicated that the date of collection affected the number of cysts per individual. Further investigation, however, into the times of migration of the sticklebacks in Tin Can Creek is necessary.

B. Encystment of Allocreadiids

Before discussing the details of encapsulation reactions to allocreadiids I shall briefly discuss the hosts and sites of encystment of the members of the Allocreadiidae.

In addition to Bunodera mediovitellata, the second intermediate host and site of encystment have been determined for a number of other members of the Allocreadiidae. Not all allocreadiids encyst in aquatic insects. Metacercariae of this family of digeneans have also been reported from bivalves and crustaceans (Table 5). In crustaceans metacercariae have generally been reported from the hemocoele or cardiac region of the host. In shrimp, metacercariae have sometimes been found in the legs and eye stalks. In bivalves, the mantle is the only site of metacercarial encystment recorded. However, as the cellular defense system of both bivalves and crustaceans differs from the system of insects (Michelson, 1977) it is more suitable to compare the defence reaction of larvae of the trichopteran P. alascensis to the reaction of other insects hosting allocreadiids.

Allocreadiids have previously been reported from four orders of insects namely the Coleoptera, Diptera, Ephemeroptera and Trichoptera. These reports are summarized in Table 6. Encysted metacercariae have been reported from the subcutaneous connective tissues and serosal membranes of chironomid larvae (Bunoderella metteri Schell; Anderson et al. 1965), the fat body and

Table 5. Allocreadiids whose second intermediate hosts are invertebrates other than insects. NM= Information not mentioned in reference.

Species of Allocreadiid	Larval stage	Hosts	Location in Hosts	Reference
Allocreadium ictaluri (Pearse)	metacercariae encysted	unionid bivalves	thick mantle edge	Seitner (1951)
Allocreadium isoporum (Looss)	metacercariae encysted	fingernail clams (Sphaerium sp.)	NM	Looss (1894)
Allocreadium lobatum (Wallin)	metacercariae progenetic	amphipods- Gammarus pseudolimnaeus Bousfield, Crangonix gracilis Smith	NM	De Guisti (1962)
Bunodera luciopercae (Muller)	metacercarie encysted	Daphnia pulex (de Geer)	body cavity- intestinal region	Wisniewski (1958)
Bunodera sacculata V An Cleave et Mueller	metacercariae encysted	Daphnia similis Claus	hemocoel adjacent to gut	Cannon (1971)
Crepidostomum cornutum (Osborn)	metacercariae encysted	crayfish- Cambarus bartoni Rhodes	cardiac region	Cheng (1957)
Orientocreadium siluri (Dubinina et Byknowskii)	metacercariae encysted ?	shrimp- Limnomysis benedeni, Mesomysis kowalewskyi	thoracic cavity abdominal region, legs, eye stalks	Bychowskii et Dubininia (1954)

Table 6. Allocreadiids whose second intermediate hosts are insects. C=Coleoptera, D= Diptera, E= Ephemeroptera, T= Trichoptera, NM= Information not mentioned in reference.

Species of Allocreadiid	Larval Stage	Hosts	Site in Host	Reference
Allocreadium alloneotenicum (Wootten)	adults	(T) Limnephilus sp. (larvae)	abdominal hemocoele	Wootten (1957)
Allocreadium neotenicum (Peters)	adults	(C) Dytiscus acilius Leach	hemocoele	Peters (1957)
Bunodera mediovitellata Zimbalik and Roytman	metacercariae encysted	(T) Lepidostoma roafi (Milne) (larvae), (T) Psychoglypha alascensis (Banks)	silk glands silk glands	Kennedy (in lit.), Caira (present study)
Bunoderella metteri Schell	metacercariae encysted	(T) Rhyacophila (larvae) (D) Chironomidae (larvae)	hemocoele, dermis, subcutaneous connective tissue, serosa	Anderson, et al. (1965)
Crepidostomum cooperi Hopkins	metacercariae encysted	(E) Hexagenia recurvata (Morgan) (naiads), (E) Polymitarcys sp. (naiads)	NM NM	Choquette (1954)
Crepidostomum farionis (Muller)	metacercariae encysted ?	(E) Ephemera danica Mull. (naiads)	fat body, abdominal muscle	Brown (1927), Crawford (1943)
Megalogonia ictaluri (Surber)	metacercariae encysted	(E) Mayfly	gills	Surber (1928)

muscle tissues of naiads of the mayfly Ephemera danica Mull. (Crepidostomum farionis (Muller); Crawford, 1943), gills of mayfly naiads (Megalogonia ictaluri (Surber); Surber, 1928) and the hemocoele of adult diving beetles, Dytiscus acilius Leach (Allocreadium neotenicum (Peters); Peters, 1957). All site recordings of allocreadiids other than B. mediovitellata from caddisflies, have been from the abdominal or thoracic hemocoele.

Thus, the finding of metacercariae of Bunodera mediovitellata encysting specifically within the silk glands of Trichoptera larvae, represents a new site of encystment for allocreadiids. The fact that encysted metacercariae were discovered in the silk glands of individuals representing two different families of Trichoptera (L. roafi- Lepidostomatidae, P. alascensis- Limnephilidae), suggests that this site is the normal area of encystment for the metacercariae of this species.

C. Encapsulation Reactions

Excluding the adults of Allocreadium neotenicum, which have not been examined in detail, all allocreadiids reported seem capable of eliciting some response from their aquatic insect hosts. The general pattern of response of the host seems to be the formation of a cellular or 'secreted' layer around the parasite followed by the deposition of a brown-yellow pigment. Unfortunately, the descriptions of the host responses are generally

lacking in detail. Brown (1927), in examining metacercariae of C. farionis from mayflies, mentions an 'outer cyst' probably of mayfly origin. He does not mention the nature of this 'outer cyst', however, his illustration shows a fibrous layer somewhat reminiscent of a hemocyte capsule around the encysted metacercariae described here in the Trichoptera larvae. Crawford (1943) mentions that as the host 'cysts' mature around the metacercariae of C. farionis, they become surrounded by a brown-yellow pigment of mayfly origin. This description is very much like a melanization reaction such as described herein around the older cysts of B. mediovitellata in the silk gland of P. alascensis. Again Crawford (1943) does not mention the structure of the host capsule, but in his drawing the metacercaria of C. farionis is surrounded by several layers of spindle shaped cells. Wootten (1957) reported finding degenerated adults of Allocreadium alloneotenicum (Wootten) which appeared as blackened amorphous masses, but still retained the shape of the worm, again suggesting the production of melanin by the caddisflies around the parasites.

The reactions of Trichoptera to their internal metazoan parasites have been discussed by very few authors, and usually only superficially. Linstow (1897) reported a host reaction to the metacercariae of three species of distomes within a number of Trichoptera larvae. Thompson (1915) mentioned a more or less opaque capsule composed of the 'amoebocytes' of the host around parasites lying

free within the hemocoele of Trichoptera and Neuroptera larvae. Brown (1933) reported an adventitious outer spherical cyst 'secreted' by the Trichoptera larvae around metacercariae of Lecithodendrium chilostomum (Mehl.) in the thoracic hemocoele of Phryganea grandis L. Burns (1961) described an unusual reaction in Dioesmoecus larvae to the penetrating cercariae of Acanthatrium oregonense Macy. He reported that the hypodermis adjacent to the penetrating cercariae thickened and turned a reddish brown; in some cases the reaction was so rapid that cercariae failed to penetrate.

Thus from the literature to date it is apparent that Trichoptera larvae and pupae have the ability to produce some sort of capsule around their digenean parasites. In addition, after some period of time most digenean parasites seem to elicit a melanization response from the trichopteran host. However, details of the reactions of Trichoptera to internal foreign objects are sketchy, and as details of encapsulation in other insects are more extensive, the cellular encapsulation reactions of P. alascensis will be discussed in terms of these reactions in other insects. I have divided the discussion of the encapsulation responses of P. alascensis into three sections including (i) time of reaction, (ii) layers of the capsules, and (iii) hemocytes taking part in the reactions.

(i) Time of Reaction

As the infected Trichoptera larvae, in which the encapsulation reactions to metacercariae were examined, were taken from the field, the ages of the capsules were not known. Consequently reaction times could not be examined. However, reactions to Epon implants in Trichoptera were examined both seven and 35 days after implantation and can be discussed here.

Recently the hemocyte response of a number of insects to non-living implants such as cellophane, araldite, and tissue implants such as Schistocerca gregaria Forsk. nerve cord, have been examined ultrastructurally, eg. in the thysanuran Thermobia domestica (Packard) (Francois, 1975), the lepidopterans Ephestia kuehneilla Zell. (Grimstone et al., 1967), and Bombyx sp. (Sato et al., 1976), and in the phasmid Clitumnus extradentatus (Schmit and Ratcliffe, 1978). All of these authors report that the capsule was complete (no more hemocytes were seen to adhere to the surface of the capsule) by the third to seventh day after implantation. In contrast, my work on the capsule in P. alascensis showed that the capsule had begun to form by the seventh day and the majority of the capsular material was laid down sometime betwen the eighth and thirty-fifth day after implantation. This comparably slow reaction time in P. alascensis may reflect several phenomena.

First, it is possible that the larvae of P. alascensis were affected by the CO_2 anesthetic. Unfortunately Zachary

et al. (1976), Brehelein et al. (1975), and Schmit and Ratcliffe (1977, 1978) do not mention their method of anesthetization, if indeed they did anesthetize the animals. Francois (1975) reported a rapid hemocyte response, but anesthetized the thysanuran for 10 min at 10°C. Salt (1960) however, reports that with caterpillars of the tomato moth Diataraxia, although they lie inert for two to three hours after anesthetization with CO_2, they completely and thickly cover eggs of the parasite Nemeritus only four hours after the latter were injected into the hemocoele. The situation however, may differ in Trichoptera.

Second, it is possible that owing to the cold maintenance temperature (10°C) for aquatic insect larvae such as P. alascensis, the metabolism of the insect is much lower than that of organisms maintained at higher temperatures, and subsequently the reaction time of the hemocytes is extended. Grimstone et al. (1967), Francois (1975), Breheleim et al. (1975), Sato et al. (1976), Zachary et al. (1976) and Schmit and Ratcliffe (1977, 1978) all maintained their animals at 25-38°C.

However, it is possible that capsule formation in the order Trichoptera is actually a much slower process than in the other orders of insects examined to date. Although the members of the order most closely related to the Trichoptera, namely the Lepidoptera (Hennig, 1969), complete the response within three days, all individuals

were maintained at 25°C. To make the results comparable it would be necessary to examine the encapsulation response of adult Trichoptera which unlike the larvae may be maintained at 25°C. Alternatively, it would be interesting to study the encapsulation response of lepidopteran larvae maintained at 10°C. In addition, it might be instructive to examine the encapsulation response of the individuals of an order that possesses aquatic larvae and is fairly closely related to the Trichoptera, namely the Neuroptera (Megaloptera).

(ii) Layers of the Capsules

Owing to the difference in nature between non-living implants such as Epon and nerve cord, and living parasites, the reactions of insects to non-living and living foreign objects must be discussed separately. In a separate section I will compare the reactions of the two.

(a) Non-Living Implants

Schmit and Ratcliffe (1978) divided the reaction of Clitumnus extradentatus to araldite fragments into two phases (1) a recognition process in which specific cell types lyse on the surface of the implant producing a localized clot formation, and (2) the accumulation of cells around the implant. In Galleria mellonella L. Schmit and Ratcliffe (1977) reported that after 20 minutes plasmatocytes adhere solely at the sites where granule cell lysis has occurred producing clumps of cells on the surface of the implant. In P. alascensis the seven day

implant showed a spotty hemocyte response demonstrating clumps of cells similar to those reported by Schmit and Ratcliffe (1977). In some areas of the implant lone nuclei were visible. In other areas of the implant, however, fully intact cells were seen lying directly against the surface of the implant. It is possible that some cell type had lysed at the areas of the hemocyte clumps, however, without ultrastructural studies it is difficult to determine whether or not the cells directly adjacent to the implant were undergoing lysis or not.

Mature capsules around implants have been reported to be composed of two layers (Breheleim et al.,1975) three layers (Grimstone et al., 1967; Schmit and Ratcliffe, 1977, 1978) and four layers (Francois, 1975). In most cases authors agree that there is an inner layer of organized flattened cells abutting an outer layer of unorganized, not so flattened cells, sometimes containing cells resembling free-circulating hemocytes. Schmit and Ratcliffe (1977, 1978), Grimstone et al. (1967) and Francois (1975) reported a layer between the flattened layer of cells and the implant. Schmit and Ratcliffe (1977) reported this layer to be composed of lysed granular cells in G. mellonella and lysing cystocytes and some granular cells in the phasmid C. extradentatus. Francois (1975) described it as a layer of cells in the process of necrosis in Thermobia domestica. Grimstone et al. (1967) reported the inner layer to be composed of unflattened cells with a large

number of dense inclusions in E. kuehniella. In addition, Francois (1975) described one layer even more proximal to the implant than the necrosing layer as a thin limiting layer, possibly melanin.

The capsule of P. alascensis was typical of the two layered capsule reported above. Two distinct layers were seen even in light microscope thick sections. These constituted an inner layer of flattened compact cells, and an outer layer of less organized less flattened cells. At both the seven and 35 day stages, no hemocytes corresponding to round free-circulating hemocytes were taking part in the outer layer of the capsule and because of the disrupted fat body tissue located on the surface of the capsule it is difficult to determine if there is evidence of lysed hemocyte material beneath the layer of flattened hemocytes.

(b) Living Parasites

Wittig (1962) and Salt (1970) have suggested that three types of encapsulation reactions involving hemocytes exist in response to parasites in insects. Firstly, there are those in which discontinuous groups of hemocytes sparsely cover parasites in clusters or plugs around their oral and/or anal regions. Such reactions have been described by Poinar and Leutenegger (1971), Vey and Gotz (1975) and usually follow the production of melanin; hemocytes may only be present for a short period of time. Salt (1970) called this reaction 'sheath capsule formation'.

The second type of encapsulation response is one in which the parasite is surrounded by irregularly shaped clumps of loosely packed hemocytes. The surface of this capsule is often sticky and irregular. This type of reaction was found by Salt (1956) around eggs of the ichneumonid Venturia canescens (Grav.) (= Nemeritis canesens) in the stick insect Carausius morosus Br.

The last type of capsule is one which Salt (1970) termed 'ordinary cellular encapsulation' as it appears to be the most frequent type of capsule. These capsules are characterized by smooth surfaces and are composed of densely packed hemocytes. According to Salt (1970) these capsules are composed of two different layers; (1) an inner layer of hemocytes, the cell walls of which are invisible, giving the layer the appearance of a continuous mass of cytoplasm with nuclei visible at intervals, and (2) an outer layer of uncoalesced cells, some round in shape and some flattened. Salt (1956) found this type of capsule around eggs and larvae of the ichneumonid V. canescens in caterpillars of the genus Diatraxia.

The reaction produced by P. alascensis to the metacercariae of B. mediovitellata corresponds most closely to the 'ordinary encapsulation reaction' type of Salt (1970). The capsule in P. alascensis has a fairly smooth appearance and at least two layers are visible; an inner layer of densely packed cells distinguishable only by the presence of chromatin granules in the nuclei,

and an outer layer of loosely packed cells.

Two layers corresponding to the 'ordinary cellular encapsulation' reaction have also been reported by several authors (Nappi and Streams, 1969; Nappi and Stoffanolo, 1971; Gibson and Berberet, 1974), however, the details given of each layer are sparse. Fortunately, Poinar et al. (1968) and Misko (1972) have examined the ultrastructure of 'ordinary cellular encapsulation' in beetles and cockroaches respectively, and consequently our knowledge of the nature of each layer in these insects is more complete.

Poinar et al (1968) described the ultrastructure of the 72 hour capsules formed by the beetles Diabrotica blateata Lec. and D. undecinopunctata (Mann) around the the nematode Filipjevivermis leipsandra as having four major regions. These included: (1) an inner region that was noncellular and partially melanized, (2) a single irregular layer of spherical necrotic cells in which nuclei and fragments of cytoplasm were visible, (3) three to four layers of densely packed cells which were extremely flattened, and whose cytoplasmic boundaries were difficult to distinguish, and (4) an outer zone of loosely attached cells resembling free hemocytes.

The description of the third layer of the capsule described by Poinar et al. (1968) seems to most closely resemble Salt's (1970) description of the inner layer of such capsules. However, in addition the authors have

described two layers more proximal to the parasite than the cellular layer. It is possible that details of these layers are only visible in electron micrographs, and that these inner layers were present, but were not seen by Salt (1970) as no ultrastructural investigation was undertaken. The fourth layer described by Poinar et al. (1968) is somewhat similar to the description of the outer layer by Salt (1970), although no flattened hemocytes with cytoplasmic boundaries were visible as reported by Poinar et al. (1968).

Misko (1972), according to Nappi (1974), studied the ultrastructure of the capsule formed by the cockroach Periplaneta americana L. around the nematode Caenorhabdites briggsae. He found that the hemocytes at the surface of the nematode were more electron dense, and contained more membrane bound inclusions than those hemocytes in the outer regions; as encapsulation progressed these membrane bound vesicles became pigmented and fused to form masses of pigment which were subsequently deposited on the parasite. The description of this layer by Misko (1972) seems consistent with the description of the first partially melanized layer of the capsules by Poinar et al. (1968). In addition Misko (1972) suggested that during the earlier stages of capsule formation the extracellular spaces at the surface of the parasite contained amorphous material in which were found free nuclei and cytoplasmic fragments of disrupted hemocytes. This layer appears to be identical

to the layer of necrotic cells (layer 2) reported by Poinar et al. (1968). In both cases nuclei and cytoplasmic fragments were visible.

From the micrographs of Misko (1972) (in Nappi, 1974), the next layer of the capsule appears to be composed of extremely flattened hemocytes whose nuclei and cytoplasmic boundaries are visible. In the micrographs no hemocytes resembling free-circulating hemocytes are visible. However, it is possible that they were merely not present in this area of the micrograph, as the area covered is small. This layer corresponds more closely to the outer layer described by Salt (1970) than does that of the outer layer described by Poinar et al. (1968).

In P. alascensis the inner layer of the non-melanized capsule stained a dark blue with polychromatic methylene blue and contained nuclei, and large vacuolar structures which stained with a pink colour. This layer is similar to that described in Salt (1970). However, without ultrastructural work it is difficult to determine whether or not the cells in this area were necrotic, or if cellular debris was present as described in the capsules of Poinar et al. (1968) and Misko (1972). The outer layer of the capsule was composed of both flattened hemocytes and cells resembling free-circulating hemocytes. Therefore, the structure is consistent with that described by Salt (1970), and is intermediate between those described by Poinar et al. (1968) and Misko (1972).

In the melanized capsules studied in P. alascensis it is only possible to say that the melanin was deposited on the inner layer of the capsule, directly in contact with the parasite. Again, without ultrastructural work it is impossible to determine whether or not electron dense vesicles as described by Misko (1972) were present in these cells.

(c) Comparison of the Reactions to Living and Non-Living Foreign Objects

The cellular encapsulation reaction of larvae of P. alascensis to Epon implants and to metacercariae of B. mediovitellata were similar in that two distinct layers to both capsules were visible. These layers included an inner layer of flattened hemocytes which were densely packed, and an outer layer of hemocytes which were not as flattened and not as densely packed. Both capsules corresponded to the 'ordinary cellular encapsulation' reaction of Salt (1970). The reactions differed in that an homogeneous substance which stained pink with polychromatic methylene blue was visible towards the inside of the capsule around metacercariae, but was not visible around Epon implants. While cells in this inner layer around the parasite appeared flattened, their cell boundaries were not as clear as those in the inner layer of the 35 day capsule around the Epon implant.

In addition, some of the capsules around metacercariae

were seen to melanize; no melanin was seen around Epon implants. It is possible, however, that melanin is not deposited until the reaction is several months old. In this case it is possible that the implants had not been in the larvae long enough for melanin to be deposited.

(iii) Hemocytes Involved in the Reaction

Although I have proposed the production of a Summary Fate Map of the hemocytes as a good method of detecting the actual order among hemocytes, no other authors have dealt with hemocytes in this manner. Hence, any comparison of their work to mine in terms of a Summary Fate Map would be confusing. Therefore in this section on hemocytes I have attempted to discuss my work in terms of the ambiguous hemocyte terminology and types presently used in the literature. The definitions of each hemocyte type as presented by Jones (1977) have been used as reference. I shall then discuss the problems with the present hemocyte classification schemes, and attempt to demonstrate how the production of a Summary Fate Map would reduce some of the inconsistencies of classification. Lastly, I shall attempt to place the cell types seen taking part in each encapsulation reaction, on the Summary Fate Map.

(a) Non-living Implants

The encapsulation response of an insect to a non-living implant seems to involve a number of different hemocyte types. In addition, the types of hemocytes taking

part in the reactions seem to vary between insects.

In examining the reactions of the phasmid Clitumnus extradentatus to implanted araldite fragments, Schmit and Ratcliffe (1978) described cystocytes, granular cells, plasmatocytes, and spherule cells taking part in the capsule formation after one hour. After four hours only granular cells and plasmatocytes were found in the capsule and these were difficult to distinguish. In contrast, Schmit and Ratcliffe (1977) suggested that after the formation of the inner layer of lysed granular cells only plasmatocytes take part in the capsule formation around implants in Galleria mellonella. Grimstone et al. (1967) did not name the cells taking part in the reaction of Ephestia kuehniella to araldite implants; however, the micrographs show cells corresponding to the granular cells of Schmit and Ratcliffe (1977). Francois (1975) mentioned three types of hemocytes taking part in the capsule in a thysanuran (Thermobia domestica). He suggested that after four hours coagulocytes, predominate. After eight hours the hemocytes taking part in the capsule formation are divided as follows: 60% coagulocytes, 30% plasmatocytes, and 10% granulocytes. Brehelien et al. (1975) reported that the majority of the cells forming layers of the capsule are granular hemocytes, though plasmatocytes are occasionally embedded in the tissue, and coagulocytes are occasionally observed in the outer capsule.

Although most authors agree that granulocytes,

plasmatocytes, and the various intermediates between these two cell types take part in formation of the bulk of the capsule, the difference between these hemocyte types is not comparable between authors. For example, in a paper on the ultrastructure of the hemocytes of Calliphora erythrocephala (Meig),Zachary and Hoffman (1973) demonstrated a cell type which they term plasmatocyte III; I find this cell type indistinguishable from the granular cell of Schmit and Ratcliffe (1978). Even within their own work Schmit and Ratcliffe (1977) did not define the terms 'granulocyte' and 'plasmatocyte' well enough for other authors to distinguish between the two.

As ultrastructural criteria such as number of packets of microtubules, number of mitochondria and presence and number of dense inclusions were used by Schmit and Ratcliffe (1978) and Francois (1975) to distinguish the various hemocyte types, it is difficult for me to correlate my work with that of these authors. Nevertheless, it appears that the usual cell type taking part in the formation of a capsule around inert implants in P. alascensis is a large cell with a fairly large nucleus, and as it appears that granules cannot be detected with methylene blue stain, it can be said that the cells taking part are either plasmatocytes, granulocytes or intermediates between the two. Even in smears of free-circulating hemocytes no cells corresponding to the cystocytes or spherule cells of Schmit and Ratcliffe (1978) were seen.

(b) Hemocytes Involved in the Reaction to Parasites

Wittig (1962) suggested that plasmatocytes were the primary hemocyte type involved in the encapsulation of parasites. This suggestion has been supported by the work of Poinar et al. (1968) and Nappi and Streams (1969) who reported that plasmatocytes enlarged and differentiated into extremely flat disc-shaped cells which they termed 'lamellocytes'. Gibson and Berberet (1974) did not name the hemocytes involved in the reaction of the alfalfa weevil Hypera postica (Gyllenhal) to eggs of the parasitoid Bathyplectes curculionis (Thompson) and it is difficult to distinguish hemocyte types from their photographs.

Lo et al. (1975) presented S.E.M. photographs of the hemocyte reaction to an opecoelid digenean encysting in mayfly naiads. Although they did not name the hemocyte types taking part in the encapsulaion reaction, their photographs show flattened hemocytes, some with filamentous pseudopodia extending to neighbouring cells.

Misko (1972) (in Nappi, 1974) presented micrographs of capsules in the cockroach P. americana around the nematode Caenorhabdites briggsae, both one hour and one week old. The hemocytes forming capsules around the one hour old infection were full of electron dense inclusions and resemble the granulocytes found around a non-living implant by Schmit and Ratcliffe (1978). In the one week capsule, the electron dense inclusions appear to have totally vanished from the elongated cells giving them the appearance of

flattened plasmatocytes. The large hemocytes participating in the capsule around the metacercariae of P. alascensis suggests that they corresponded to the plasmatocytes of Jones (1977).

Owing to the difficulties of fixing the metacercarial cysts, I was unable to distinguish hemocyte types from the electron micrographs of the capsule around metacercariae of B. mediovitellata. However, the elongated nature of the cells seen taking part in the reaction to the established metacercariae in light microscope sections (longer than type #5 in Figure 13) is suggestive of a plasmatocyte-like cell. That the cells responding to the young metacercariae were not as extended as the cells responding to the established metacercariae may reflect two facts: (1) the hemocytes (plasmatocytes?) which respond to the parasite, flatten as the encapsulation proceeds, or (2) the difference in fixation between the two capsules affects the details of the hemocytes preserved. The young metacerarial capsules were fixed with Bouin's fixative whereas the established metacercariae were fixed with glutaraldehyde; the glutaraldehyde is a much better fixative as it preserves the details and extensions of cells to a greater extent than does Bouin's.

(c) Summary Fate Map as a Solution

Various authors have distinguished from one (Moran, 1971) to fourteen (Yeager, 1945) types of isect hemocytes from various orders of insects. This diversity of opinion

stems from the fact that researchers in the past, have been unable to determine if hemocyte types are morphological representations of phases in the life of a blood cell, each phase with a different function (single cell theory), or several distinct types of cells which are immutable and serve different roles in insect physiology (multiple cell theory). According to Arnold (1979), three main sources of this controversy exist: (1) the inadequacy of our definition of hemocyte type - there are no cytological characters that distinguish each with certainty in all insects - at least not to the satisfaction of different authors, (2) the differences in the hemocyte types are not homologous in different groups, and (3) the tendency of hemocytes to react differently to changes in their environment, and thus to different techniques used to prepare them.

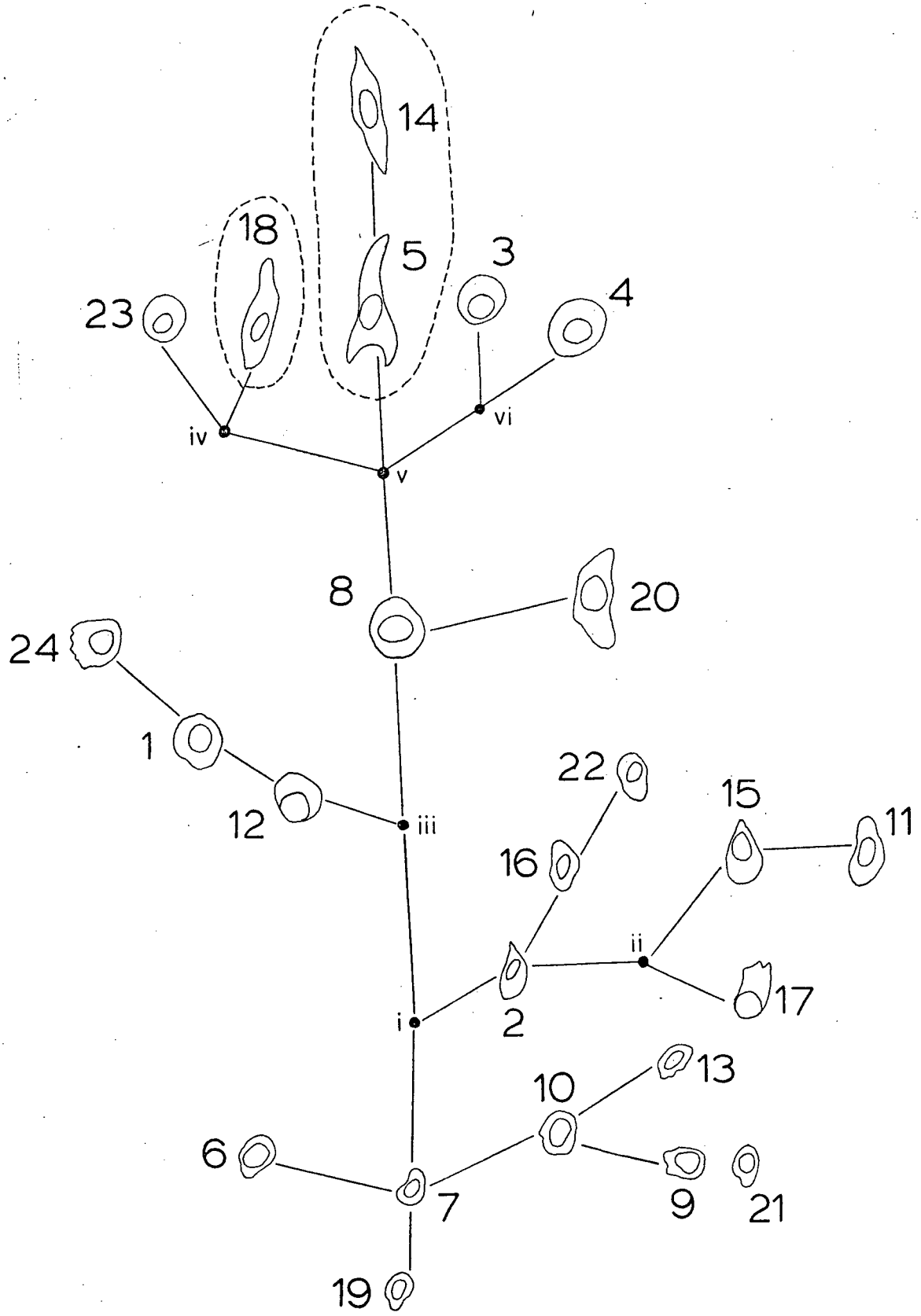
What is lacking in the hemocyte work, then, is an objective method of recognizing any consistent general pattern of relationship among the various hemocytes and subsequently producing a general reference system. In this study I have attempted to produce such a reference system of hemocyte classification using quantitative parsimony analysis (Farris, 1970) of morphological data for a number of hemocytes from P. alascensis. This method of analysis was originally developed as an aid to reconstructing phylogenies (cladistics), but has been generalized as the method for producing the most efficient,

least ambiguous summations of any data set (Farris, 1979).

In order to ensure that the hemocytes were strictly comparable I examined only hemocytes heat-fixed and then giemsa-stained. The cladistic analysis led to the production of a consistent pattern of relationship among the hemocytes examined. This pattern was heirarchical. At the base of the pattern was a small cell with a small nucleus/cytoplasm ratio, and a dense nucleus, this cell corresponds to the prohemocyte or suggested precursory cell of Jones (1977) and Arnold (1979). Because the pattern produced is a heirarchical one, it follows that the further out on the branching diagram you go, the more traits the hemocytes at this point share. Each trait represents a progressive transformation of a character from the prohemocyte state of that character. Many of the various transformations are represented in the observed cells. Therefore, because each of the observed cells represents some subset of those transformations, then the more transformations two cells share in terms of the cladogram, the more likely it is that they are derived from a common set of precursor cells. In other words the cladogram may be viewed as a Summary Fate Map indicating both the ontogenetic potential available to a particular cell and the ontogenetic constraints placed on a cell by progressive specialization. For example in Figure 27 cell #2 could potentially become #16, 22, ●(ii), 15, 11, 17, or any of their intermediates. It could not however, become cell #3, for example. The idea that hemocytes pass

FIGURE 27

Summary Fate Map (Figure 13). Lineages of hemocyte types implicated in participation in encapsulation of non-living and living objects in Trichoptera are circled.


14
18
5
3
4
23
iv
vi
v
8
20
24
1
12
iii
22
15
11
16
ii
17
2
i
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6
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9
21
19

from one developmental stage to another is supported by the work of many authors (Crossley, 1964; Hoffman, 1966; Hoffman et al., 1968; Moran, 1971; Price and Ratcliffe, 1974).

Authors up to now have been trying to put each cell into a 'type' class when in fact each observed cell simply represents the progression of the ontogeny of an individual cell to a particular point. The entire ontogeny for cell #24 is 19→7→●(i)→●(iii)→12→1→24. But for cell #20 the entire ontogeny is 19→7→●(i)→●(iii)→8→20. If we want to understand the common developmental elements we would use that designation which would show us that 19→7→●(i)→●(iii) is common to the ontogeny of both #24 and 20. However in terms of understanding the progressive differentiation of #24 and 20 it is more informative to consider the restricted lineages 12→1→24 and 8→20 respectively. Thus the Summary Fate Map is useful for examining both similarities and differences However, there are some problems in classifying hemocytes, for example, observing a cell which corresponds to ●(iii) in Figure 27 does not tell us if that cell would have developed into a cell like #12 or 8. Therefore if we recognize #24 and 20 as representing different specialized cell lineages and therefore requiring differential classification, we would have to place cells conforming to # ●(iii) in the category containing #24 and in the category containing #20. In any classification, therefore, the only cells which can be unambiguously classified are

the non-subdividing lineages. In other words in Figure 27, cells #19, 6, 12→1→24, 23, 18, 5→14, 3, 4, 20, 16→22, 15→11, 17, 13, 9 and 21 could be classified. The other cells including 2, 7, 8, 10, ●(i), ●(ii), ●(iii), ●(iv), ●(v), and ●(vi), would have to be included in at least two different categories in the classification. In the case of these cells then, the determination of the position of these cells in the Summary Fate Map, as totipotent cells, rather than classifying them as cell 'types' would lead to much less confusion than attempting to classify them as cell 'types'.

Although non-subdividing lineages may be classified they should not be classified in terms of each cell in the lineage. Rather these cells should be classified in terms of the lineage itself, as each cell in this lineage represents part of a transformation series, it is less confusing and more exact. Only if the lineage is composed of a single cell with one character state change can this cell be given a type name. For example in Figure 27 cells # 12, 1 and 24 would be considered as one type of hemocyte lineage.

If the present classification scheme of hemocyte types such as that described by Jones (1977) is a natural scheme, my suggestion of a method of classification will not eliminate the terms used in this scheme, but will merely extend them to a lineage of hemocyte types so that we will speak in terms of the 'granulocyte lineage'

and the 'plasmatocyte lineage'. If however, the present method of classification is not a natural scheme, for example if the cells presently termed plasmatocytes do not represent a single lineage of cells, then such terms will have to be altered or dropped altogether from the classification scheme.

In order to determine the robustness of the hemocyte terms now in common usage, cells classified using these terms should be compared to the cells in the Summary Fate Map. Unfortunately, not much work has been done on the hemocytes of Trichoptera and therefore it is difficult for me to compare the hemocyte lineages of the Summary Fate Map to a published classification scheme of Trichoptera hemocytes. Price and Ratcliffe (1974) examined the hemocytes of the limnephilid Anabolia nervosa and reported that the larvae possessed prohemocytes, plasmatocytes, granulocytes, cystocytes and possible oenocytoids. The authors, however, did not publish photographs of any of these hemocyte 'types' and thus no comparison of these hemocytes with the Summary Fate Map can be made.

Throughout this study then, I have dealt with individual hemocyte types rather than hemocyte lineages and attempted to correlate them with the classification scheme of Jones (1977), since dealing with lineages would make the study non-comparable to those of others. At this point, it is however possible to speculate as to which lineages of hemocytes the cells seen taking part in

the encapsulation responses may belong. From the lengths of the cells seen taking part in the reaction to the seven and 35 day implants, as well as to the established metacercariae, it appears that these cells correspond most closely to cells #18, 5 and 14. This suggests that the cells participating in the encapsulation reaction may have developed from two lineages of cells. These cell lines are circled in Figure 27. That cells even more elongated than types #18, 5 and 14 were found in the capsules suggests that these elongated hemocytes are subsequent stages in the development along these two lineages of cells.

D. Hemocyte Activation

At present the mechanism of hemocyte activation remains unclear. Salt (1970) maintained that the main question to be examined is whether the hemocytes are attracted to a foreign object from a distance or whether the hemocytes make contact with the surface of an object during their circulation and are subsequently stimulated to adhere. Salt (1970) favoured the former explanation. He suggests that if hemocytes were seen to flatten before their actual contact with the capsule it would show a stimulus had reached them before they fortuitously contacted the parasite, but he suggests that this has not been seen. However, precocious flattening of hemocytes has been observed in dipterans by Nappi and

Streams (1969) and Nappi and Stoffanolo (1971).

Several theories as to the nature of a possible stimulus attracting hemocytes to the parasite have been suggested. Nappi (1974) questioned how any object introduced into the body of an insect could avoid contamination from substances released from tissues injured during the entry of the body, and subsequently attract hemocytes. In fact, Cherbas (1973) identified an injury factor which she called 'hemokinin' from epidermal cells and the hemolymph of saturniid pupae. She suggested that hemokinin may also be associated with other cells such as hemocytes.

A second theory maintained by Nappi (1974), is that a change in the normal titre of host hormones is brought about by parasitization. Such changes alter the permeability of cell membranes to certain metabolites and bring about precocious differentiation and migration of hemocytes.

In the present study I have examined a situation in which a parasite has moved to an area, the lumen of the silk glands, where hemocytes are not normally present. Further, the cellular defense system of the caddisfly was tested by implanting pieces of Epon into the hemocoele, an area where hemcoytes are plentiful.

Had hemocytes not responded to the parasites in the lumen of the silk glands, but only to implants located within the hemocoele, it would have been evidence in support of the 'random bumping' hypothesis of Salt (1970). However, hemocytes did respond to the parasite within the lumen of the

silk gland. Although this suggests that the hemocytes are being drawn into an area where they would not normally go, it tells us nothing about which stimulus the hemocytes are responding to. In this case it could be: (1) secretions (hemokinin?) from the damaged epithelial cells of the silk gland, (2) substances released from other cells that were damaged by the parasite as it moved from the cuticle into the silk gland, (3) substances released from the metacercariae, or (4) a change in the titre of host hormones resulting from parasitism.

E. Wound Healing

The fact that the epithelial layer of the silk gland of P. alascensis around metacercariae of B. mediovitellata was totally replaced by hemocytes has so far been discussed in terms of a defensive cellular encapsulation reaction directed at the parasite. However, since it is possible that the hemocytes were responding to the epithelial cells of the silk gland which had been damaged by the encystment of the metacercariae within the lumen of the gland, rather than the parasite itself, it is now appropriate to discuss the role of hemocytes in the wound healing reactions of insects.

In the past, wound healing has been examined in the assassin bug Rhodnius prolixus Stål. (Wigglesworth, 1937; Locke, 1966; Lai-Fook, 1968, 1970), the gut of the cockroach Periplaneta americana (Day, 1952), the gut of the mosquito

Aedes aegypti (L.) and the leafhopper Orosium argentatus (Ev.) (Day and Bennetts, 1953), and caterpillars of the wax moth Galleria mellonella (Rowley and Ratcliffe, 1978). In several cases hemocytes were not involved in the wound healing reaction. Wigglesworth (1937) and Day and Bennetts (1953) found that hemocytes would not respond to cuticular wounds unless the wound was more than superficial, and the basement membrane was damaged. However, epithelial cells responded normally. In addition, Day and Bennetts (1953) demonstrated that healing occurred in the gut of A. aegypti without the presence of hemocytes. The authors attributed this to the rapid regenerative powers of the gut epithelium.

Nevertheless, in most insects so far examined, hemocytes have been involved to some extent in the repair of injury. In most cases hemocytes were reported to form a plug over the wound thus preventing the loss of material from the gut (Day, 1952; Day and Bennetts, 1953) or hemocoele (Wigglesworth,1937; Lai-Fook, 1968, 1970; Rowley and Ratcliffe, 1978). The plug so formed appeared to be a temporary structure, present only until the epithelial layer was able to migrate over the hemcoytes and re-establish itself (Wigglesworth, 1937; Day, 1952; Day and Bennetts, 1953; Locke, 1966; Lai-Fook, 1968, 1970). Wigglesworth (1937) suggested that after the epithelial cells had crossed over and sealed the wound, the hemocytes forming the plug would dissolve and disappear. Day (1952) demonstrated that when the epithelium had completely re-established its

continuity, the 'wound tissue' formed of hemocytes was reduced. He suggested that this was because hemocytes had left the wound and begun to circulate again.

In their study Rowley and Ratcliffe (1978) suggested that four hemocyte types were involved in the wound healing reaction of Galleria mellonella these included: plasmatocytes, granular cells, spherule cells and oenocytoids. They suggested that plasmatocytes and granular cells were the most abundant of the cell types. The other authors did not mention the types of hemocytes involved in the wound healing reactions.

In my opinion then, three characteristics of the hemocyte reaction in P. alascensis suggest that it is an encapsulation rather than a wound healing reaction: (1) in all cases of wound healing so far examined the hemocytes form a temporary plug until the epithelial cells take over; in no slides of the reaction of P. alascensis were signs of regeneration of the silk gland epithelium visible. It appeared that rather than forming a temporary wound plug until the epithelium was able to regenerate itself, the hemocytes took on the shape of the silk gland epithelium and remained as a permanent structure around the parasite. Even in the older metacercarial infections which had been melanized, hemocytes were well organized and no sign of epithelial regeneration was visible; (2) although Ratcliffe and Rowley (1978) demonstrated the formation of a capsule-like structure around the

damaged area in G. mellonella the structure was unorganized and not like the organized two layered capsule seen in P. alascensis; (3) in a normal wound healing reaction hemocytes move to an area where tissue has been damaged and temporarily take the place of these damaged tissues. In the silk glands of P. alascensis hemocytes were seen, not only to replace the epithelial layer of the silk gland, but also to enter into the lumen of the gland and flatten against areas of the parasites which had never been in contact with damaged tissue.

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VI. APPENDIX

Protocol for Spurrs Sections

FIXATION:	2.5% glutaraldehyde, .05 M sodium cacodylate, 1% sucrose, .01 M calcium chloride, 1 drop diluted photoflow.	overnight in vacuum
WASH:	5% sucrose, .05 M sodium cacodylate, 1 drop diluted photoflow.....	overnight in vacuum
POST FIX:	1% osmium tetroxide, .05 M sodium cacodylate, 2% sucrose..	6 hrs in vacuum
EN-BLOCK STAINING:	Distilled water.....	2x 10 min
	2.5% uranyl acetate in distilled water.....	2 hrs in dark
DEHYDRATION:	35% ethanol.....	15 min
	50% ethanol.....	15 min
	70% ethanol.....	15 min
	80% ethanol.....	15 min
	90% ethanol.....	15 min
	100% ethanol.....	2x 1 hr
	100% ethanol: propylene oxide 3:1.....	overnight in vacuum
	etOH:PO- 2:1.....	overnight in vacuum
	etOH:PO- 1:1.....	overnight in vacuum
	etOH:PO- 1:2.....	overnight in vacuum
	etOH:PO- 1:3.....	overnight in vacuum
	100% PO.....	2x 30 min in vacuum
INFILTRATION:	PO:resin- 3:1.....	overnight in vacuum
	PO:resin- 2:1.....	overnight in vacuum
	PO:resin- 1:1.....	overnight in vacuum

VI. APPENDIX continued

	PO:resin- 1:2.....	overnight in vacuum
	PO:resin- 1:3.....	overnight in vacuum
	100% resin.....	overnight in vacuum
EMBEDDING:	100% resin in beam capsule....	8hrs, 60°C in 15lbs vacuum