A STUDY ON THE BLOOD PROTOZOA OF BLUE GROUSE ON VANCOUVER ISLAND

by

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B.Sc. University of British Columbia, 1962

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF

Master of Science

in the Department

of

Zoology

We accept this thesis as conforming to the required standard

The University of British Columbia

September, 1964.

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ABSTRACT

The present study demonstrates that blue grouse on Vancouver Island are infected with two species of *Haemoproteus*, probably two species of *Leucocytozoon* and a species of *Trypanosoma*.

Haemoproteus dendragapi n.sp. is described from the Nanaimo Lakes Area. The growth rate of *H. canachites* gametocytes is much more rapid than that described by Fallis in Ontario. The very young tissue stages of *H. canachites* are described from lung preparations of grouse chicks.

The life cycle of *Leucocytozoon bonasae* has been completed by using a new vector, *Cnephia minus*. As reported by Fallis in Ontario, *Simulium aureum* has been found to be a vector of *L. bonasae* on Vancouver Island. This study has verified Woodcock's often ignored hypothesis that the morphology of the gametocyte-host cell complex changes with age of infection. A probable new species of *Leucocytozoon* is described from the Campbell River Area.

In-vitro culture of the trypanosome from grouse blood has been carried out. A yearling blue grouse has been successfully infected by inoculation of metacyclic trypansomes from the culture.

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. J.R. Adams for his constant encouragement, enthusiastic guidance, and critical supervision during the entire phase of this work.

Thanks are due to Drs. C.V. Finnegan and J.F. Bendell for their helpful suggestions during the preparation of the manuscript. The author wishes to thank Dr. C.V. Finnegan for the use of his photographic equipment.

The author is indebted to Dr. P.C.C. Garnham, Professor of Protozoology, London School of Hygiene, London, for a critical review of some of the work. Thanks are also due to Dr. A.M. Fallis, Department of Parasitology, Ontario Research Foundation, for his critical comments and also encouragements.

Throughout all phases of the work, the author had greatly benefited from the assistance of others. This is particularly true of the collection of live infected birds and also 'clean' grouse chicks. To Messrs. F.C. Zwickel, J.Dixon, G.G. Gibson and L.J. Russell the author is truly grateful for their assistance in the field.

To Mr. T. Barsby the author wishes to express his gratitude for the use of his cabin in the Nanaimo Lakes Area during the summer of 1964.

The author also wishes to thank Mr. Arthur Ko, Dept. of Bacteriology, for his assistance in making up the culture medium for the in-vitro studies.

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INTRODUCTION

This research project was undertaken as an attempt to identify the parasitic blood protozoa of the blue grouse, Dendragapus obscurus fuliginosus, (Ridgway), in Vancouver Island.

Fowle (1946) reported the occurrence of Haemoproteus sp., Leucocytozoon sp., and Trypanosoma sp. from two localities in Vancouver Island. He gave figures on the incidence and the degree of infection in 44 birds examined in 1943-44. Adams and Bendell (1953) indicated that of 252 adult birds examined from Campbell River, Vancouver Island, Haemoproteus occurred in 92%, Leucocytozoon in 87%, and Trypanosoma in 76%. Bendell (1955) reported similar high protozoan infections in yearling blue grouse. His study was carried out in the spring and summer of 1950, 1951, and 1952. Of the 174 yearlings examined, 169 had Haemoproteus (97%), 148 had Leucocytozoon (85%), and 134 had Trypanosoma (70%). Chicks examined during this same period showed a lower percentage of infection (Haemoproteus 66%, Leucocytozoon 38%, and Trypanosoma 20%).

None of these previous workers had attempted to identify these blood protozoa to species. The main purposes of this thesis are:

(1) to establish the identities of the blood parasites in the Vancouver Island blue grouse, and to describe new species if they occur.

(2) to identify the vectors responsible for the transmission of these parastiic protozoa, and also the time of transmission.

Parasitic blood protozoa have been recorded from various grouse. The four most frequently reported genera are (a) Haemoproteus, (b) Leucocytozoon, (c) Plasmodium, and (d) Trypanosoma.

(a) Haemoproteus

The earliest record of Haemoproteus in grouse was that of Sambon (1908). He named it Haemoproteus mansoni, but no description was given. Fatham (1910) working with red grouse (Lagopus scoticus) again recorded H. mansoni; he found what he thought were young stages and a very brief description was given. Clarke (1938), and Fallis (1945) reported a halter-shaped Haemoproteus from ruffed grouse in Ontario. Fallis and Bennett (1960) described and named a halter-shaped species from spruce grouse (Canachites canadensis) H. canachites. In British Columbia, Haemoproteus infections in blue grouse were reported by Fowle (1946), Adams and Bendell (1953), and Bendell (1955); but none of these reports indicate what species was present.

Fallis and Bennett (1960) studied the stages of sporogony of H. canachites in Culicoides sphagnumensis Williams, and were able to infect 'clean' ruffed grouse chicks to complete the life cycle. They found that the mature gametocytes exflagellated within three minutes after they were ingested by Culicoides, and that the zygotes were present in the

stomach twelve hours after ingestion. These average 5.5μ in diameter. Oocysts (averaging 6.3μ) were present in the stomach wall four days after ingestion, and one day later developing sporozoites were seen in a few oocysts. By the sixth day after ingestion of the blood meal many oocysts contained sporozoites. Thirty oocysts averaged 9.5μ and contained residual bodies 3-4μ in diameter.

The development of the tissue stages in the grouse was not followed or described by these authors.

In the genus Haemoproteus the gametocytes occur in the red blood cells and are usually halter shaped. Schizogony takes place in the lungs, and not in the erythrocytes. The known vectors are louse-flies (Huff 1932) and midges (Fallis and Wood 1957; Fallis and Bennett 1960). A synonym of this generic name is Halteridium Labbe, 1894. Members of this genus are extremely common in wild birds and also in domestic pigeons, ducks and turkeys. Coatney (1936) gave a check list and host-index of the species of Haemoproteus, and Herman (1944) listed the species reported from North American birds.

(b) Leucocytozoon

Leucocytozoon lovati Seligmann and Sambon 1908 was first described from the red grouse (Lagopus scoticus) by Sambon (1908). Fantham (1910) gave a fuller description of the mature gametocyte stages and he also described the immature gametocyte stages. Clarke (1935) described Leucocytozoon bonasae from ruffed grouse (Bonasa umbellus) and spruce grouse (Canachites canadensis) in Algonquin Park,

Ontario. He had earlier noted the occurrence of this parasite in Algonquin Park, and its association with a heavy mortality of young birds (Clarke 1934). Following its description, L.bonasae was recorded by Fallis (1945) and Erickson (1953) from ruffed grouse. Cowan and Peterle (1957) reported it in Michigan sharp tailed grouse.

No attempt to solve the life cycle was made until 1958 when Fallis and Bennett (1958) successfully infected 'clean' [X] ruffed grouse chicks by injecting a saline suspension of sporozoites from black flies that had previously fed on infected grouse. The vectors responsible for the transmission of L.bonasae in Algonquin Park are Simulium latipes and S. aureum. Fallis and Bennett (1962) followed the sporogony of L. bonasae in black flies and compared the various stages with those from L.mirandae Franca 1912 and L. fringillinarum Woodcock 1910. They found that the sporogony of some individuals was completed in less than five days at 22°C±2; others required nine days or more. Oocysts of L. mirandae averaged 14μ (9-19) in diameter, those of L. bonasae 13μ (8-16), and those of L. fringillinarum 11μ (8-16). No measurements were given for the size of the sporozoites except that it was noted that the sporozoites of L. mirandae were slightly longer and narrower than those of the other two species and more numerous in the larger oocysts.

Fallis and Bennett (1958) had noted that the gametocytes of L. bonasae circulating in the blood of the bird were of two forms: one in which the gametocyte is in a host cell

[X] 'clean' grouse: a grouse that has been raised as a chick in an indoor cage and the blood found to be negative for blood protozoa prior to infection.

that has attenuated ends, the other form in a host cell that lacks the attenuated ends. They indicated that this is possibly due to a difference in gametocyte age.

In the genus Leucocytozoon the macrogametocyte and the microgametocyte occur in the leucocytes or, in some species, also in the erythrocytes. Schizogony takes place in the parenchyma of the liver, heart, kidney or other organs, the schizonts forming large bodies divided into cytomeres. There is no schizogony in the erythrocytes or leucocytes. The vectors are black flies (O'Roke 1934; Fallis, Anderson and Bennett 1956; Skidmore 1932; and Fallis and Bennett 1958). Leucocytozoon is common in many wild birds and is the cause of disease in ducks, geese, turkeys and chckens. Coatney (1937) gave a catalogue and host-index of the genus, and Herman (1944) listed the species occurring in North American birds.

(c) Plasmodium

Wetmore (1939) was the first to report a Plasmodium from the prairie sharp-tailed grouse. This grouse plasmodium is characterised by the elongated gametocytes that displace the host cell nucleus slightly when mature by producing from four to twelve merozoites. No identification of the Plasmodium was given in the paper. Fallis (1945) reported a species of Plasmodium with characters resembling those of P. circumflexum in ruffed grouse. Experimental results published a year later (Fallis 1946) identify this Plasmodium to be a strain of P. circuflexum, and he referred to it as the grouse Plasmodium. Dorney and Todd (1960) in a survey of the spring incidence of ruffed grouse blood parasites in Wisconsin also noted

Plasmodium infection. The percentage of infection is very low throughout the year.

About forty species of Plasmodium have been described from birds, but only fourteen or fifteen are accepted as valid (Hewitt 1940; Bray 1957; Laird and Lari 1958). Many wild birds are commonly infected. Herman (1944) and Coatney and Roudabush (1949) have given catalogues and host-indices of the species of Plasmodium in birds. The chief characteristics of this genus are, the gametocytes occur in the erythrocytes, schizogony takes place in the erythrocytes and also in various other tissues.

All the three genera mentioned so far, belong to the sub-order Haemosporina. Chief characteristics of this suborder are: the zygote is motile, schizogony takes place in the vertebrate host, and sporogony in the invertebrate. If the erythrocytes are invaded, pigment (hemozoin) is formed from the host cell hemoglobin.

The gametocytes of both the genera Haemoproteus and Plasmodium occur in the erythrocytes. These gametocytes can be either round or elongated. Plasmodium is distinguished from Haemoproteus by the fact that schizogony takes place in the erythrocytes to produce merozoites; while in Haemoproteus schizogony is restricted to the parenchyma of internal organs. Hence artificial transmission of Plasmodium is possible by blood innoculation while Haemoproteus can be experimentally transmitted only by tissue transplants or by innoculation of tissue emulsion.

(d) Trypanosoma

Trypanosomes have been reported under a large number of names from many species of birds. They all look very much alike and probably belong to relatively few species. Extensive cross transmission studies are needed to establish their relationships. They are however referred to in this thesis by the names under which they were first described.

Trypanosoma avium Danilewsky, 1885 was first described from owls (specific name not given) and roller-birds (Coracias garrulus) in Europe, and has since been reported from a wide variety of birds including crows (Baker, 1956) and Canada geese (Diamond and Herman 1954). Baker (1956 a, b) transmitted it from the rook (Corvus fruglegus) and the jackdaw (C. monedula) to canaries, but failed to transmit it to a single 3-day-old chick.

T. calmettei Mathis and Leger, 1909 was described from the chicken in South-East Asia; it is about 36µ long. T. gallinarum Bruce et al., 1911 was described from the chicken in Central Africa as about 60 long. T. hannai was described from the pigeon, and T. numidae from the guinea fowl.

Avian trypanosomes are very polymorphic, sometimes attaining great size. They may be 26µ to 60µ long or even longer. The kinetoplast is generally a long distance from the posterior end. There is a free flagellum, and the body is often striated.

Blood-sucking arthropods such as mosquitoes and

hippoboscids are believed to be vectors of avian trypanosomes, but the only complete life cycle was worked out by Baker (1956, a, b) for T. avium from rooks and jackdaws. He found that in England the hippoboscid fly, Ornithomyia avicularia, acts as the vector. Upon ingestion with the blood meal, the trypanosomes change into the crithidial form in the mid-gut, multiply by binary fission in this form, and pass to the hind-gut. They multiply further and then turn into a piriform stage which develops into a small metacyclic trypanosome form. Birds become infected when they eat infected insects. The metacyclic trypanosomes penetrate the membrances of the mouth, oesophagus and/or crop and probably invade the lymphatic system, developing into large forms which first appear in the blood 18 to 24 hours after infection.

According to Baker (1956), there is no multiplication in the avian host, the trypanosomes simply become larger. This could account for their sparse numbers in the blood. They persist in the rook and jackdaw over winter, being more or less restricted to the bone marrow, and reappear in the peripherial blood in the spring. Diamond and Herman (1954), too, found that T. avium could be isolated from the bone marrow of Canada geese much more readily than from the blood.

Nothing is known of the pathogenicity of the avian trypanosomes. They are presumably non-pathogenic.

Bennett (1961) reported T. avium from a great variety of birds in Algonquin Park in Ontario. His conclusions were that T. avium has a posterior station in the fly vector, and

experiments indicated that the parasite is probably transmitted by the penetration of flagellates (in the feces of infected flies) through breaks in the host skin.

MATERIALS AND METHODS

Blood Smear Preparation and Staining

Blood smears for microscopic studies of the gametocyte stages were made from blood taken from the brachial vein of live birds; blood from dead birds was taken from the lungs. The smears were air dried as rapidly as possible to prevent any change in the form of the parasite before the smears have dried, since it is known that mature microgametocytes of Leucocytozoon may exflagellate within a minute at room temperature (Fallis and Bennett 1958) and immature Leucocytozoon and Haemoproteus gametocytes may round up when the blood temperature is lowered (Woodcock 1910).

For quick diagnosis of parasitic infection in the field, dried blood films were stained with Wright's Stain for two minutes, after which an equal volume of buffered distilled water (pH. 7.0) was added and let stand for another 2-3 minutes. This was flushed off with water and the smear dried and examined under medium power for trypanosomes and Leucocytozoon. For Haemoproteus the smear was examined under oil.

For detailed study of parasite morphology the smears were first fixed in 100% methyl alcohol for one to two minutes, dried and then stained with Giemsa Stain for approximately forty-five minutes. The saline Giemsa technique (Shute and Maryon 1961) was slightly modified by this author for better differentiation. Sodium chloride (0.5 gm) was dissolved in one litre of buffered distilled water (pH 7.2).

The staining medium is made up of 0.8 cc. of "Gurrs Improved Giemsa Stain (R66)" in 45 cc. of 0.005% NaCl buffered distilled water. The smears were stained for at least forty minutes after which no change occurred. Smears left in the stain for twenty-four hours appeared as if they have been stained for forty minutes.

Study of Tissue Stages (Schizogony in Host Tissues)

Three different approaches were used in studying the tissue stages of Leucocytozoon and Haemoproteus in the host bird. They were:

1. Organ Smear.
2. Organ Impression.
3. Organ Section.

1. Organ Smear. A minute piece of host tissue (e.g. liver, spleen or lung) was mashed on a clean slide, at the same time spreading it as thinly as possible. It was then fixed in 100% methyl alcohol and stained in Giemsa's stain as described earlier.

2. Organ Impression. This was made by imprinting the freshly cut surfaces of an organ on to a clean slide. This was fixed in 100% methyl alcohol and stained as previously described.

3. Organ Section. The method used was that of Shortt and Cooper (1947) with slight modifications. Fresh organs taken from birds were cut into pieces two to three centimeters thick and fixed immediately in Carnoy's Fixative for approximately twenty-four hours.

Carnoy's Fixative:

Glacial Acetic Acid	30 cc.
Chloroform	30 cc.
Absolute Alcohol	30 cc.

The organ slices were then transferred to 95% alcohol and finally to 70% alcohol where they were preserved until sectioning. The organ slices were sectioned at four to five microns thick. In the staining technique, difficulty in dissolving the colophonium in acetone to the recommended concentration was overcome by first dissolving the colophonium in as little absolute alcohol as possible (colophonium is very soluble in absolute alcohol), and adding this to the acetone. Also 2 cc. of "Gurr's Giemsa" was used instead of the 10 cc. as recommended.

In-vitro Culture of Trypanosoma

In-vitro culture of trypanosomes from the blue grouse was carried out in the diphasic medium used by Tobie, von Brand, and Mehlman (1950) for the cultivation of T. gambiense and T. rhodesiense. This medium is composed of a rich blood agar base (solid phase) and a liquid phase in which the trypanosomes divide.

The culture medium was first incubated at 37C for twenty-four hours to ensure that the medium was free of bacterial contamination. Blood from the infected grouse (S4-64) was then inoculated into the liquid phase of the medium. The whole procedure was carried out as aseptically as possible. To further ensure that the cultures were free of bacterial

contaminations 1500 units of penicillin was added to each of the culture tubes.

The cultures were incubated at 25°C for six days and checked every twenty-four hours to follow the course of the dividing forms in the cultures. These forms were studied in live preparations as well as in fixed and stained smears. The smears were heat dried and then stained with Giemsa's stain.

After six days of incubation at 25°C, four of the six cultures were incubated at 38°C, while two cultures were left at 25°C. The cultures were left at their respective new temperatures for another four days checking every twenty-four hours for signs of bacterial contaminations.

On the tenth day of incubation three of the 38°C cultures were inoculated intra-peritoneally into a 'clean' grouse yearling and two young domestic chicks (four weeks old). Also one of the 25°C cultures was injected into a four week old domestic chick.

The experimental birds were checked for the presence of S-shaped trypanosomes by direct blood examinations and by stained preparations. This was done twenty-four and forty-eight hours after infection. Also blood from these inoculated birds was cultured at 25°C to confirm infection.

Field Methods:- Capture of Birds

Live infected blue grouse were caught with a long pole (12-15 feet) that has a loose noose at one end. This was usually done with the help of a trained dog. Grouse chicks were obtained from the wild broods a few days after they had

hatched when their capture was feasible, usually during the last two weeks of June and the first two weeks of July. Following capture, blood smears were made from each of them to determine whether they were infected prior to capture. Blood smears were made at regular intervals from infected chicks to follow the course of infection.

Exposure Method (Anderson 1956)

The infected bird was placed in a cage (14"x14"x14"). A hood, made of dark cloth placed over the head of the exposed bird keeps the bird relatively quiet in the exposure cage so that flies are able to feed on it without being dislodged. The bird in the exposure cage can be raised to various heights by means of a rope.

The cage with the hooded bird was raised to the desired height in ten to fifteen seconds and left there for approximately thirty minutes. It was then lowered carefully (30-40 seconds) on to a plywood square ($2\frac{1}{2}$'x$2\frac{1}{2}$') following which it was covered with a collecting cage which consisted of a frame (20"x20"x20") covered with screens on four sides; the top being covered with a plastic sheet and the bottom left open. Strips of rubber sponge glued to the bottom frame of the cage form a tight seal and prevent the escape of flies. The bird was placed in the collecting cage for approximately thirty minutes. Engorged flies which had left the bird and settled on the side of the cage were collected in aspirators through a sleeve at the side of the collecting cage.

Birds were exposed at the Nanaimo Lakes Area during the period June 12th to July 1st. Unpublished records from previous years (Gibson) show this period to be the height of the black fly season in this area. The birds were usually exposed in the evenings between 6.30 and 9.30 p.m. at a height of forty feet, but on very windy evenings the exposure height was fifteen feet.

The engorged flies were kept in small cages (Anderson 1956) in which a high humidity and free water were provided by wet paper towels placed at the bottom of the cage.

Longevity of the flies was presumably extended by reducing the light and the rate of evaporation in the cages by means of loosely fitting covers. Flies were fed daily on dilute honey. Specimens were dissected in 0.85% saline at regular intervals after their engorgement (Shute and Maryon 1960), heat fixed and then placed in absolute methyl alcohol. This dissolved the sodium chloride crystals before the preparations were stained in Giemsa.

Heads and thoraces of flies, in which sporozoites were presumed to be present were emulsified in 0.85% saline and injected subcutaneously into a 'clean' yearling blue grouse. Fly emulsions were also injected with saline into one week old domestic chicks. As controls, some birds were injected with saline. Blood smears were prepared from all experimental birds to determine if they had become infected, and if so, to follow the infection in the blood.

RESULTS

Studies of blue grouse chicks, yearlings, and adults from three localities on Vancouver Island during the summers of 1963 and 1964 have confirmed previous records of Fowle (1946), Adams and Bendell (1953), and Bendell (1955), that Vancouver Island blue grouse are infected with blood protozoa of three genera, namely Haemoproteus, Leucocytozoon, and Trypanosoma.

Detailed studies of the blood smears of birds from each of these localities indicate that there are two species of Haemoproteus, probably two species of Leucocytozoon and one species of Trypanosoma.

Haemoproteus

Of the two species of Haemoproteus, one has been identified as H. canachites Fallis and Bennett 1960. This identification is based purely on the morphological similarities of the gametocyte stages as found in the blood of our Vancouver Island blue grouse and the species H. canachities described by Fallis in Ontario. This species is common to all birds in the three study areas. The percentage of infection of yearling and adult birds during the spring and summer of both 1963 and 1964 was about 98% (Table I). Blood smears of chicks made from the 1963 Game Check at Campbell River indicate only 26.9% infected at the end of August (Table II). However a chick caught in late August near Courtenay started to show the infection on August 29th, and a detailed description of the various stages of gametocyte formation was obtained.

TABLE I

Summary of Parasitic Blood Protozoa observed in Adult and Yearling Blue Grouse (1963-64)

Date	Locality	Sample Size	Infected Birds		
			Haemoproteus	Leucocytozoon	Trypanosoma
June 1963	Middle Quinsam Lake	11	11	11	10
July 1963	Middle Quinsam Lake	9	9	9	9
	Courtenay	12	12	12	10
August 1963	Middle Quinsam Lake	6	6	6	5
March 1964	Courtenay	1	0	1	1
May 1964	Courtenay	3	2	3	3
June 1964	Nanaimo Lake Area	20	20	20	16
	TOTAL:	62	60	62	54
	% Infected		96.8%	100%	83.9%

The other Haemoproteus recorded from the Vancouver Island blue grouse during the study period is a new species. The name Haemoproteus dendragapi is proposed for it. The range of this new species seems rather limited. It occurs in grouse in the Nanaimo Lakes Area, but birds from the other two study areas are infected only with H. canachites. Pure infection of H. dendragapi is found in grouse up Black Jack Ridge Nanaimo Lakes Area (3 out of 4). Blue grouse that are found in the flats below Black Jack Ridge have a mixed infection of H. canachites and H. dendragapi (18 out of 18).

Haemoproteus dendragapi n. sp. (Figures 4 and 5)

Host: Dendragapus obscurus fuliginosus. (Ridgway)

Locality: Black Jack Mountain, Nanaimo Lakes Area, Vancouver Island.

Slides used in the following description are A9, A10, D22.

EARLY GAMETOCYTES

Form and Size: Spherical or slightly ovate, appearing as single hyaline objects between the nucleus of the red blood cell and its periphery. Size about 1-1.5 μ.

Double infection in a single host cell is not uncommon.

Nucleus: Not differentiated, represented by a peripheral blob of staining red chromatin.

Cytoplasm: Clear, periphery darker with occasional reticular areas which stain pale blue.

Vacuole: A small clear area occupying almost the entire organism

Pigment Granule: Absent.

IMMATURE GAMETOCYTES (Figure 4)

Form and Size: Spherical or slightly ovate bodies, usually located at one end of the long axis of the red blood cell nucleus. Average size 1.5-4.5 μ diameter; slightly displacing the nucleus of the red blood cell to one side in older stages. Male gametocyte staining blue while female stains intensely blue.

Nucleus: More definitely recognizable as a distinct structure. Usually more centrally located in older stages. Oval to spherical in shape and staining red especially around the periphery. Nuclear membrane not sharply defined.

Cytoplasm: Varying from clear to granular, blue staining reaction much more pronounced than in earlier stages.

Vacuole: Vacuole now diffuse or broken up into 2-3 smaller vacoules; diffused or absent in older stages.

Pigment Granule: From 3-16, occurring in groups of 2-4. May be irregularly scattered, approaching those of the adult gametocyte in size.

MATURE GAMETOCYTE (Figure 5)

Male Gametocyte

Form and Size: Slightly ovate to spherical; dislodging the red blood cell nucleus completely to one side and frequently occupying the former space of the red blood cell cytoplasm completely. Length 6.84-11.2 μ Width 5.75-8.04 μ

Nucleus: Ovate to spherical, can be anywhere in the parasite cytoplasm. Stains pink in Giemsa. Average size 1.5-3.5 μ becoming more diffused. Karyosome not usually seen.

Cytoplasm: Pale, almost hyaline.

Vacuole: Very diffused and sometimes indistinct.

Pigment Granule: Minimum number 19, maximum number 30; tendency to be in groups of 3-5; shape from spherical to rod-like; carbon black in appearance, highly refractile.

Host Cell: Enlarged; and only the host cell nucleus can be seen, the space formerly occupied by host cell protoplasm is all taken up by the parasite gametocyte.

Female Gametocyte

Form and Size: Like male gametocyte; ovate to spherical dislodging red blood cell nucleus to one side, hence greatly disfiguring the red blood cell. Measurements in general are the same as for male gametocyte, but there is greater tendency to produce greater hypertrophy of the red blood cell.

Nucleus: Spherical to oval, more compact than that of male gametocyte. Average size 1-2.5μ diameter, stains dark pink to red; karyosome frequently distinct and is usually on the periphery of the nucleus.

Cytoplasm: Easily distinguishable from that of male gametocyte by the darker staining. Reticular appearance quite distinct.

Vacuole: Not commonly seen.

Pigment Granule: As in male gametocyte.

Host Cell: As in microgametocyte.

Detailed Description of the Immature Stages of H. canachites

Blue grouse chick, "NOBAND II", was approximately 45 days old when it was captured on August 22nd, in the Middle Quinsam Area, Vancouver Island. It was brought back to Vancouver on August 23rd, and put into an indoor cage. Blood smears made on August 23rd, 25th, and 27th, showed it to be free of

blood protozoa. On August 29th at 10.00 A.M. a fourth blood smear was made. This smear has very young ring stages between the nucleus of the red blood cell and its periphery. They look not unlike the ring stages of Plasmodium and in fact they were mistaken to be such at that time. Twenty-one of such rings are measured. Most of them are slightly oval, the average length being 2.24μ and the width being 1.46μ ; the diameter of the parasite nucleus is about 0.56μ . This nucleus is just a peripheral blob of chromatin staining red. The cytoplasm is clear, the periphery is darker with occasional reticular areas which stain pale blue. Pigment granules are absent. The vacuole is a small clear area.

The fifth smear was made at 2.00 P.M. on August 29th. This smear, made four hours after the previous smear, shows an increase in the trophozoite size; these older trophozoites are quite elongated. Eighteen specimens are measured and the average length is 4.02μ and the width 2.8μ . The nucleus is more distinct and measures about 1.2μ in diameter. A number of these trophozoites (7 out of 18) has yellow pigment in the parasite cytoplasm, and in some there are 2 or 3 dark pigment granules along with the yellow ones.

The sixth smear was made at 10.00 P.M. on August 29th. In this smear the gametocytes have grown to about full size. Male and female gametocytes can be distinguished by their staining reactions. The female gametocytes stain dark blue and in some the karyosome can be identified . The male gametocytes stain light blue and the nucleus is rather

elongated and diffused. Though these gametocytes have grown to adult size they can be distinguished from the sexually matured ones, by being highly vacuolated, by still having yellow pigments along with very fine dark pigment granules.

The seventh smear made on September 2nd shows these gametozoites to be fully matured. Vacuolation has disappeared, the yellow pigments have given way to the usual dark refractile pigment granules.

Tissue Stages of Haemoproteus canachites (Figure 10)

Lung smears (K1; and K26) made from two blue grouse chicks (field number 63-6; and 63-16 respectively) show very young tissue stages of H. canachites. These smears were made at the 1963 Game Check held at Campbell River (Table II). Smear K1 shows a very light Haemoproteus infection, while K26 is negative for blood stages. Because of the immaturity of the tissue stages, the host cells can be identified as belonging to the macrophage system. Manwell (1961) and Kudo (1960) have stated that the tissue stages of Haemoproteus occur mainly in the endothelial system of the lungs.

The youngest stage from these smears is a minute uninucleate body within the cytoplasm of the host cell. This minute uninucleate body is composed of a single red chromatin dot with dark blue staining cytoplasm. This probably represents the first stage after the entry of the sporozoite into the host cell. As the parasite grows, the red staining nucleus starts dividing until there are about twelve of these distinct nuclear masses. At this phase of growth, division

TABLE II

Game Check Blood Smears - Blue Grouse Chicks, 1963.

Field No.	Slide No.	Locality	H.	L.	T.	Remarks
63-6	K1	Beaver Lk.	✓	✓	✓	Tissue states of Haemoproteus; attenuated L. bonasae.
63-9	K2	Gooseneck Lk.	✓	-	-	Heavy infection of H. canachites.
63-11	K3	Bacon Lk.	-	✓	✓	Heavy infection of L.bonasae(attenuated)
63-15	K4	Menzies Mt.	-	-	-	Negative.
63-18	K5	L.Quinsam Lk.	-	✓	✓	Heavy infection of L.bonasae(attenuated)
63-30	K6	Merrill Lk.	-	✓	-	Heavy infection of L.bonasae(attenuated)
63-32	K7	Camp V	-	✓	✓	Attenuated L. bonasae
63-34	K8	Mohum Lk.	✓	✓	✓	Young of H.canachites; attenuated Leucocytozoon.
63-38	K9	Mohum Lk.	-	✓	✓	Attenuated L. bonasae.*
63-49	K10	Campbell Lk.	-	✓	-	Attenuated L. bonasae.
63-56	K12	Campbell Lk.	✓	✓	✓	Attenuated L. bonasae.*
63-63	K13	Camp V.	-	✓	-	Leucocytozoon"A" and L. bonasae.
63-67	K14	Gooseneck Lk.	-	✓	✓	Leucocytozoon"A" and L. bonasae.
63-73	K15	Patterson Lk.	-	✓	✓	Light infection of L. bonasae.
63-74	K16	Gooseneck Lk.	✓	✓	✓	Light Haemoproteus and L. bonasae.
63-82	K17	M.Quinsam Lk.	-	✓	-	Leucocytozoon "A" and L. bonasae.
63-102	K18	Berry Creek	✓	✓	✓	Light Haemoproteus and L. bonasae.
63-107	K19	Camp V.		✓	✓	Attenuated L. bonasae.
63-108	K20	Camp V.	✓	✓	✓	Leucocytozoon "A" and L. bonasae.
	K22			✓	✓	Attenuated L. bonasae *
63-27	K23	L.Quinsam Lk.		✓	✓	Attenuated L. bonasae *
63-12	K24	Bacon Lk.		✓	✓	Round L. bonasae.
	K25			✓	✓	Light L. bonasae (attenuated).
	K26			✓	✓	Leucocytozoon"A" and L. bonasae, also tissue stages of Haemoproteus.

Percentage infection of chicks at the end of August, 1963:

Leucocytozoon $^{24}/_{26}$ x 100 = 92.3%

Haemoproteus $^{7}/_{26}$ x 100 = 26.9%

Trypanosoma $^{18}/_{26}$ x 100 = 70%

of the nuclear masses stops, while growth in size continues. Each of these nuclear masses is now called a cytomere. The oldest stage found so far is the twelve cytomere stage in which five of the twelve cytomeres show sign of budding. These five cytomeres represent the beginning of the next and final stage in the schizogony of the parasite.

Discussion

Haemoproteus dendragapi (Figures 4 and 5) in spite of its round gametocytes is considered a Haemoproteus because no schizonts were ever detected in blood smears of 21 birds that harboured the infection. These birds were killed at various times of day over a period of 25 days in June. Also the staining reaction of H. dendragapi is identical to that of H. canachites (Figure 3) (which has the usual halter gametocytes). Thirdly, the presence of refractile pigment granules that are similar to those of H. canachites make it more of a Haemoproteus. Fourthly H. sacharovi Novy and MacNeal, 1905, a Haemoproteus with rounded gametocytes has been confirmed by Huff (1932) who worked out the life cycle.

The most prominent feature of H. dendragapi is the manner in which the mature gametocyte pushes the nucleus of the red blood to one side so that the latter appears to sit on the parasite as a cap. This species differs greatly from H. canachites in form and size. All the gametocyte stages, from immature to adult forms, are round while the immature forms of H. canachites are elongated and the adult gametocytes halter shape. The number of pigment granules in the adult

gametocytes of both species is approximately the same.

H. dendragapi resembles H. sacharovi in that both have round gametocytes which push the red blood cell nuclei to one side. They differ greatly in size (H. dendragapi is smaller than H. sacharovi) in the number of pigment granules (H. dendragapi has many more pigment granules than H.sacharovi), and finally in host bird (H. dendragapi is described from blue grouse and H. sacharovi from mourning doves).

Results from Noband II show that the trophozoites of H. canachites from our Vancouver Island blue grouse grow to the adult gametocyte stage within 12 hours. This growth rate is far greater than that reported by Fallis and Bennett (1960) for H. canachites in their experimentally infected ruffed grouse. According to Fallis and Bennett (1960) it takes approximately 6 days for the trophozoite to grow to mature size. This great difference in the rate of growth of the gametocyte is hard to account for. Various factors which might contribute towards this great difference in growth rate have been considered but none seemed satisfactory. The smear made on August 27th (two days before ring stages were seen) was carefully examined under an oil immersion lens for stages younger than those recorded from the smear made on the 29th, at 10.00 A.M., but no parasite could be found. In fact none of the smears made before the 29th show any sign of an infection.

The adult gametocytes of H. canachites from the ruffed grouse in Ontario are very similar if not identical to the halter shape H. canachites found in our blue grouse. Because of the great similarities of the gametocytes our halter shape Haemoproteus has been tentatively identified as H. canachites. Further investigation by cross infections and the study of tissue stages should confirm its identity.

The young tissue stages from the lung smears of 2 chicks were identified as those of H. canachites; they resemble the illustrations and descriptions of the tissue stages of H. columbae in Kudo's "Protozoology". The tissue stages cannot possibly be those of Leucocytozoon because in Leucocytozoon there is always a central body present in a developing megaloschizont (Cowan 1955). Also, megaloschizonts of Leucocytozoon develop mainly in the spleen, heart, and liver (Cowan 1955); and the tissue stages of Haemoproteus develop mainly in the endothelial cells of the lungs (Kudo 1960, Manwell 1961). This author has repeatedly found fully grown Haemoproteus schizonts from the lungs of juncos.

As confirmation, tissue stages similar to those described from the chicks have also been recorded from the lung smear of a yearling shot in early June 1964 at the Nanaimo Lakes Area. A blood smear from this particular yearling also shows very young ring stages in the red blood cells.

Leucocytozoon

Various forms of Leucocytozoon were encountered in blood smears from birds during the study period. Figures 6, 7,

8, and 9 are photomicrographs of the gametocytes of Leucocytozoon that are found in the circulating blood. Figure 1 shows the basis on which they were measured.

Initially it was not known whether these forms were variants of a single species or different species, and only through a study of sequence of blood smears from infected birds, by comparisons of smears from birds in different localities and by life cycle studies were the final conclusions reached.

As stated earlier, two species of Leucocytozoon are present in the Vancouver Island blue grouse. Of the two species identified in this study, one of them corresponds closely to the description of Leucocytozoon bonasae Clarke 1935. Hence it is referred to as such in this thesis. Sixty two yearling and adult blue grouse, examined from the three study areas between 1963 and 1964, were all positives for this form (100% infection, Table I). Blood smears of chicks from the 1963 Game Check (Table II) also show a very high percentage of infection (92.3%). All of five chicks shot on July 23rd near Courtenay were positive for L. bonasae. These chicks were all from different broods in the same general area. Another one of these chicks was caught and brought back to the laboratory where the course of infection of the blood stages was studied.

The other Leucocytozoon species recorded in this study resembles the description of L. fringillinarum Woodcock 1910, in form only. It is distinctly 2-3 longer. Since only

sexually matured forms were found, it is difficult to make a positive identification. It is in all likelihood a new species, but until more is known of its various blood stages and the vectors responsible for its transmission, it will tentatively be referred to as Leucocytozoon 'A'.

Leucocytozoon 'A' seems quite localized. It has only been found in the Campbell River Area. Blood smears of chicks from the Game Check show that about 20% were infected with this Leucocytozoon (5 out of 26; Table II). No pure infection of Leucocytozoon 'A'has been recorded so far, it usually comes as a mixed infection with Leucocytozoon bonasae. It has not been recorded from yearlings and adults in the Campbell River Area during the summers of 1963 and 1964.

Life Cycle of Leucocytozoon bonasae

An adult female blue grouse (S4-64) caught on May 28th, 1964 in Courtenay was used as the exposure bird. Blood smears made before and after the exposure period (June 13th - July 1st) showed it to be infected with L. bonasae, H.canachites, and trypanosomes. Table V is a field record of the number and species of engorged flies collected from the exposure bird during the exposure periods at the Nanaimo Lake Area in Vancouver Island.

The most abundant ornithophlic black flies in this area during the exposure period were Simulium aureum and Cnehpia minus. For all practical purposes all black flies collected from the exposure bird are considered as belonging to one of these two species.

Engorged Simulium aureum and Cnephia minus dissected 12 hours after collection had both zygotes and motile ookinetes (Table III). The zygotes average 12.4 μ in diameter; the ookinetes are crescent shaped with one end narrower and more pointed than the other. The nucleus stains pale red and the cytoplasm pale blue. The nucleus, which is slightly oval, is located more towards the pointed end. There are 2-3 vacuoles in the cytoplasm. The average length of the ookinetes is 26 μ.

Salivary glands dissected out of the head of both S. aureum and C. minus 10 days after collection from the exposure grouse had sporozoites. The sporozoites are about 10.4 μ in length and 1.68 μ in width. The nucleus which is slightly elongated (1.68x1.12 μ) stains red while the cytoplasm stains pale blue. One end of the sporozoite is more pointed than the other, and the nucleus is located more to the rounded end.

On June 28th the heads and thoraces of two C. minus were emulsified in 0.85% saline and injected intra-peritoneally into a 'clean' yearling blue grouse (Table IV). The 2 C.minus were collected from the exposure grouse 10 days previously (on June 19th).

Blood smears made from this yearling grouse 3½ weeks later were positive for L. bonasae, thus completing the life cycle. Attempts to infect domestic chicks with L. bonasae were made, (Table IV). Ten 2 week old domestic chicks were inoculated with infected fly suspensions at various times during July.

TABLE III

Parasite Stages recovered from Dissected Flies after Engorgement

Fly Species Dissected	Time after Engorgement	Parasite Stage Found	Site of Parasite Stage
Cnephia minus	1 hour	L.bonasae Exflagellation Figure 13	Stomach of fly
Cnephia minus Simulium aureum	12 hours	L.bonasae zygotes ookinetes Figures 14 & 15	Stomach of fly
Simulium aureum Cnephia minus	5-6 days	leptomonad stages of trypanosome	Fore gut and vicinity of salivary gland
Simulium aureum	10 days	L. bonasae sporozoites Figure 16	Salivary gland
Cnephia minus	10 days	L. bonasae sporozoites Figure 16	Salivary gland

TABLE IV

Summary of Experimental Transmission of *L. bonasae* to Blue Grouse and Domestic Chicks

No. of Birds	Experimental Bird	Age of Bird	Species of Flies injected	No. of Flies injected to each bird	Time since Flies fed on injected grouse	Date Injected	Remarks
1	Blue grouse	Yearling	*C. minus*	2	10 days	June 28	Gametocytes in circulating blood after 25 days
3	Domestic chick	15 days	*C. minus*	2	9 days	July 6	Negative
2	Domestic chick	17 days	*S. aureum*	3	10 days	July 8	Negative
2	Domestic chick	21 days	*S. aureum*	4	8 days	July 12	Negative
2	Domestic chick	24 days	*C. minus*	2	9 days	July 14th	Negative

TABLE V

Record of Engorged Flies collected from Exposure Bird
(June 13-July 1, 1964)

Date of Exposure	Time of Exposure	Weather Condition	Number and Species of Flies (engorged) collected
June			
13th	1900-2030	Very windy 55°F	4 Culicoides 2 C. minus
14th	---------	Rain	----------
15th	1830-2030	Windy 57°F	8 Culicoides 4 C. minus 8 S.aureum
16th	---------	Rain	----------
17th	---------	Rain	----------
18th	1830-2030	Calm, very humid, 68°F	15 Culicoides 6 C. minus 19 S.aureum
19th	1830-2030	Calm, humid 65°F	21 Culicoides 8 C. minus 9 S.aureum
20th	1830-2030	Calm 70°F	17 Culicoides 10 C. minus 16 S.aureum
21st	1900-2030	Very windy 56°F	3 Culicoides 2 C. minus 4 S.aureum
26th	1800-2100	Calm 65°F	14 Culicoides 9 C. minus 15 S.aureum
27th	1830-2130	Windy 67°F	5 Culicoides 5 C. minus 6 S.aureum
28th	1900-2130	Calm 69°F	16 Culicoides 9 C. minus 8 S.aureum
29th	1830-2130	Calm 70°F	18 Culicoides 13 C. minus 6 S.aureum
30th	1830-2130	Calm 71°F	21 Culicoides 13 C. minus 3 S.aureum
July			
1st	1830-2130	Calm 70°F	20 Culicoides 15 C. minus 3 S.aureum

TABLE VI

Comparison of Various Sizes of *Leucocytozoon* Gametocytes

Parasite	Form	Sample Size	Parasite Length (μ)	Parasite Width (μ)	Host Nucleus Length (μ)	Host Nucleus Width (μ)	Parasite-Host cell complex length (μ)
Leucocytozoon bonasae	Attenuated Form Type I	26	21.84-23.52	5.60-3.92	10.08-14.00	3.92-2.80	31.36-34.72
	Figure 6						
	Attenuated Form Type II	25	14.56-19.16	11.76-7.84	10.08-14.00	3.92-1.68	25.20-28.56
	Figure 7						
	Round Form	30	9.52-11.76	11.76-8.96	8.96-13.44	3.92-1.68	---
	Figure 8						
Leucocytozoon 'A'	Round Form	35	11.20-12.88	11.76-8.40	6.16-8.40	6.16-4.48	---
	Figure 9						

Some were inoculated with C. minus suspensions and some with S.aureum suspensions. Blood smears from these chicks were negative for parasites. This would tend to indicate that L. bonasae is quite host specific, since both the grouse and the domestic chicken are galliform birds.

Relationship between Gametocyte Age and Parasite-Host Cell Shape in Leucocytozoon.

Fallis and Bennett (1958) had noted that the gametocytes of L. bonasae circulating in the blood of the bird were of two forms. One, in which the gametocyte is in a host cell that has attenuated ends, the other in a host cell that lacks the attenuated ends. They indicated that this might possibly be due to a difference in gametocyte age.

In the present study these different gametocyte forms were again recorded. The two forms were especially prominent in adults and yearlings in May and early June. Chicks shot in late June (5 out of 5) at the Courtenay study area had only the attenuated forms, while chicks examined later on in the summer had both forms. In studying through the series of blood smears from about thirty chicks collected between July and August (most of the smears are from the 1963 Game Check, Table II) it was noticed that there was considerable variation in length and width measurements of both the parasite itself and the parasite-host cell complex in the attenuated form. For convenience in descriptions the attenuated forms are arbitrary sub-divided into two types.

Type I:- Where the parasite-host cell complex measures over 30 μ; and the parasite itself is oval. (Figure 6 and Table VI).

Type II:- Where the parasite-host cell complex is less than 30 μ in length; and the parasite itself is less oval, approaching the shape of the round form. (Figure 7 and Table VI).

Gametocytes that are described as belonging to the round form (Figure 8) in this thesis are quite round; and are found in host cells in which the attenuation is visually lacking. The host cell nucleus stains red in Giemsa's stain and is laterally compressed to one side of the parasite-host complex. In most of the specimens studied, the original host cell membranes can be seen as loosely fitting envelopes around the parasites.

"Noband I" was captured near Courtenay on July 23rd, 1963. The approximate age at the time of capture was about 30 days. A blood smear made at the time of capture indicated that it was infected with L. bonasae and trypanosomes. Blood smears were made on subsequent days to follow the course of infection of L. bonasae.

On slide H63, made on July 23rd, the whole smear contained only twelve L. bonasae gametocytes, all of which are of the attenuated forms. In ten of these, the parasite itself is very oval (average length 22.54 μ, width 4.64 μ), and the whole parasite-host cell complex is on the average 32.84 μ. In the other two specimens the parasite itself is

less oval (average length 17.46μ width 7.94μ) while the parasite-host cell complex averages 26.74μ in length. Hence the gametocytes just described can be classified under the two categories of the attenuated forms proposed earlier as ten of Type I and two of Type II.

In slide J2 made on July 30th (made 7 days after H63) most of the attenuated forms are of Type II. Altogether there are twenty two attenuated forms, of these only five of the specimens are of Type I, the remainder seventeen belong to Type II.

On August 2nd a third smear was made (slide J7). In this smear only Type II of the attenuated forms are seen. Also appearing in the smear are round forms in which the host cell attenuation is visually lacking. Of the nineteen specimens counted, four are of the round form. The average parasite length in the round form is 10.12μ and the width is 9.24μ.

The last smear was made on August 5th, just before the chick died. In this smear (slide J16) there are predominantly round forms; twenty one of these specimens are round forms while only five are Type II of the attenuated form.

Autopsy of the chick showed it to be heavily infected with Dispharynx (13) and the proventriculus was badly damaged and swollen. It was forced fed since August 1st when it stopped eating.

TABLE VII

Sequential Smears showing the Relationship between Gametocyte Age and Form in Leucocytozoon bonasae.

Date	Slide Number	Total No. of Gametocytes	Attenuated Forms		Round Forms
			Type I	Type II	
July 23	H63	12	10	2	0
July 30	J2	22	5	17	0
August 2	J7	19	0	15	4
August 4	J16	26	0	5	21

Description of Leucocytozoon 'A' (Figure 9).

As mentioned earlier in the introduction, this is in all probability a new species, but as only sexually mature forms were found it is felt that this is insufficient data for the description of a new species.

Leucocytozoon 'A' has been recorded only from blue grouse chicks in the Campbell River Area. Records from the 1963 Game Check at Campbell River show that six of twenty-six chicks (23%) in a random sample from this area are infected with this species. Blood smears of chicks, yearlings, and adults from the other two study areas are negative for this particular Leucocytozoon.

The following is a description of the species based on the measurements of thirty-five specimens. In Table VI the measurements of this species are compared with those of L. bonasae Clarke, which occurs in all of the three study areas in Vancouver Island.

Leucocytozoon 'A' resembles L. fringillinarum Woodcock 1910 in that both parasites are round and that the host cell lacks attenuation, it differs from L.fringillinarum in size. More important is the fact that in both parasites the host cell nucleus is triangular and sits on top of the parasite like a 'cap'. Microgametocytes are noticeably less frequent than macrogametocytes. The cytoplasm of the macrogametocyte is dark blue (Giemsa's Stain), and is quite granular and vacuolated; that of the microgametocyte stains

pale blue and is more uniform. The nucleus of the macrogametocyte stains pale blue and is more uniform. The nucleus of the macrogametocyte stains pale red and at its periphery there is usually a darker staining blob of chromatin, which has been identified as a karyosome (Clarke 1935; Atchely 1951). The nucleus is more or less round, about 2.0μ or 2.5μ in size, and can be located anywhere within the cytoplasm of the parasite. The nucleus of the microgametocyte is pale red, oval, and is usually centrally located. It is more diffused and is about 9x3.5μ in size. The parasites themselves are more or less round and measure 11.20-12.88μ by 11.76-8.40μ (Table VI). As mentioned earlier the host cell lacks attenuations. Its nucleus is a dark red triangular body which lies to one side. The length of the host cell nucleus is 6.16-8.40μ , and the height is 4.48-6.16μ (Table II).

Discussion

In working out the life cycle of Leucocytozoon bonasae Fallis and Bennett (1958) found that the vectors responsible for transmission of the parasite in Algonquin Park, Ontario, are Simulium aureum and S. latipes.

Transmission studies of L. bonasae, in the Vancouver Island blue grouse, were carried out in the Nanaimo Lakes Area during the period June 13th to July 1st. Field records show that the most prevalent species of ornithophlic black flies during this period are Simulium aureum and Cnephia minus. Dissection of these engorged flies indicated that sporogony

of L. bonasae takes place in both these species of black flies as sporozoites were recovered from the salivary glands of dissected flies that had previously bitten the exposure bird. C. minus as a new vector for L. bonasae is further substantiated by the fact that a 'clean' yearling blue grouse was infected with L. bonasae after it was inoculated with a saline suspension of the heads and thoraces of two C. minus that had bitten the exposure bird 10 days previously.

An analysis of the field record shows that of the two species of black flies recovered from the exposure bird, S. aureum is the more abundant in mid-June and that C. minus is more prevalent in late June and possibly early July. Since both species can act as vectors, this would mean that S.aureum is the more important vector in June, and C. minus is responsible for transmission in July. Hence chicks that hatch out during June and July would have a good chance of being infected by one or both the vectors. This is not the only instance where transmission of a parasite at different times of the summer is carried out by different species of vectors. Fallis, Anderson and Bennett (1956) working with the transmission of L. simondi found that S. croxtoni and S. euryadiniculum are the important vectors during the early part of the black fly season (May to June) in Ontario while S. rugglesi is the important vector in late June and July.

Results obtained from studies of the successive blood smears (Table VII) made from "Noband I" indicate that

the gametocytes in the attenuated host cells are younger than those that are in host cells that seem to lack the attenuation. This is shown by the fact that blood smears made at the beginning of the study period had mostly attenuated forms and those that were made at later dates had all round forms (which incidentally do not seem to show any attenuation). Since attenuated forms have more oval gametocytes than the round forms it can be said that young gametocytes are oval and that old gametocytes are round. This observation seems to agree with the view expressed by Woodcock (1910) that authors "in describing the gametocytes (and their host cells) as rounded, appear to have been dealing simply with individuals which had begun the active process of rounding themselves off, preparatory to rupturing the host cell and becoming liberated as ripe gametocytes".

Studies on the young gametocyte stages of L. bonasae also help to substantiate the above conclusion. Only very young stages (those that are less than 5μ in diameter) are round. As they grow (to about 11.15μ) they become oval and they start to cause attenuation of the host cell. Also from studies of very young gametocytes it is concluded that mononuclear leucocytes are the host cells and that monocytes are more parasitised than lymphocytes.

The fact that only round gametocytes are found in adult birds in late summer, and early spring can now be explained as the remnants of the young gametocytes of the

previous spring. Also some of the chicks from the Game Checks and some of the chicks shot in July and August had only the attenuated forms (e.g. 63-22, 63-49 etc. Table II); this can be explained by the fact that the infection was a recent one and that the gametocytes had no time to grow "old".

These observations would tend to refute the theory that the round and attenuated forms are the results of sporozoites and/or merozoites parasitizing different host cells. According to the reasonings of this theory one should not expect to find birds with pure infections of either one form as the proportions of different white blood cells in any one animal should remain relatively constant. But if it is accepted that the attenuated forms are the younger stages of the round forms, then it is possible to find birds infected with only one form. A bird with a pure infection of attenuated forms would indicate that the infection was a recent one; this is especially true in chicks collected in late summer. Also adult birds with a pure infection of round forms might indicate that it did not have a recent relapse and that all the gametocytes in the blood were "old".

A possible explanation of the relationship between the age of the gametocyte and the shape of the whole parasite-host cell complex is that as the young oval parasite grows, more of the host cell cytoplasm is used up, and as this happens the attenuation on either side becomes less pronounced. This goes on until all or most of the host cell cytoplasm is

used up. When this happens all or most of the space that originally occupied by the host cytoplasm is now occupied by the parasite, or alternatively this empty space does not show up in stained specimens. The author has in many instances noticed a very loose host cell envelope around the gametocyte.

Leucocytozoon 'A' which has been described as a probable new species is a round form (i.e. both the gametocyte and the host cell are round), not unlike that of the old mature forms of L. bonasae. In fact the size range of the two overlaps quite considerably. The striking difference between the two is the shape of the host nucleus. In Leucocytozoon 'A' the host nucleus is triangular and sits on top of the parasite like a cap while in the old mature form of L. bonasae the host nucleus is only a narrow strip of chromatin on one side of the parasite.

The only other round Leucocytozoon that has been described as having a triangular host nucleus sitting on it, is L. fringillinarum Woodcock 1910. The chief difference between these two species is in their size (Leucocytozoon 'A' is at least 2-3μ longer than L. fringillinarum). Furthermore these two are found in different families of bird host. L. fringillinarum is described from the finches while Leucocytozoon 'A' is from blue grouse. Attempts by this author to infect domestic chicks with L. bonasae have failed; and Fallis, Pearson, and Bennett (1953) were not able to infect ruffed grouse, chickens, turkeys, and pheasants with L. simondi of

of ducks. These experiments seem to indicate that Leucocytozoon species are quite host specific, and that L. fringillinarum infecting a blue grouse is highly improbable. Hence it may be said that Leucocytozoon 'A', though it may resemble L. fringillinarum in form, is a distinct species.

Trypanosoma.

In the present studies, trypanosomes have been found in small numbers in blood smears of 54 adult and yearling blue grouse out of a total of 62 examined from the three study areas in Vancouver Island during the summers of 1963 and 1964. The percentage infection of adult and yearling birds is 84%, which is slightly higher than that reported by Adams and Bendell (1953). As for chicks, the percentage infection is very much higher than that reported by previous workers. Blood smears from chicks made at the 1963 Game Check at Campbell River showed a 70% infection (Table II), whereas Adams and Bendell (1953) reported a 17% infection in chicks.

All specimens observed from blood smears were in the large S-shaped stage described by Novy and MacNeal (1905) as the most mature. In all, nineteen specimens were measured; the basis on which the measurements were made are shown in Figure 2. The specimens measure 50.5-55μ long (average 53.2μ) and 4.5-6μ (average 5μ wide). The kinetoplast is slightly oval (1.5x1μ) and lies about 12.6μ from the posterior end. It stains quite dark red with Giemsa Stain. The rim of the undulating membrane stains pink and is plainly visible along

most of the body length. The free flagellum appears to be rather long, about 5-7μ. The nucleus is rounded and stains pale red. It lies on the outer curvature of the body. In diameter it is approximately 4μ, the size varying with the degree of flattening of the specimen. The cytoplasm stains dark blue. A number of specimens show myonemes, the number of these vary from 5-7. In some specimens, these myonemes can be seen running parallel to each other along most of the length of the body.

The lengths and some of the other measurements seem to fit those of T. gallinarum Bruce et al. 1911 (Table IX), but as trypanosomes in general are very polymorphic the cultural characteristics should also be considered in the identification.

Blood from an infected bird was cultured in the diphasic medium employed by Tobie, von Brand, and Mehlman (1950) for the study T. gambiense and T. rhodesiense. It was incubated at 25°C for a period of six days. No dividing forms were ever detected in the culture in the first twenty-four hours. After forty-eight hours incubation at 25°C scanty slender leptomonad forms were observed. These forms average 8.8μ long and 1.5μ wide; the flagellum is approximately 7μ long. Some of the leptomonad forms show a slight bulge near the anterior end; in stained specimens this area seems to be occupied by the nucleus and the kinetoplast, both staining red in Giemsa's Stain. The cytoplasm is vacuolated and stains pale blue.

By the third day of incubation the culture was growing very well, still only leptomonad stages were seen. On the fourth day the whole culture was teeming with long slender leptomonad stages, but there were also some shorter and rounder forms which seemed to be the dividing forms. The long slender forms had also started to agglutinate. This begins with two cells sticking together at their posterior ends which slightly overlap. In the living preparations the line of junction cannot be made out, and as a result the double cell appears to be a single organism with a whip at each end. Also, 'triplets' were present, in these it is assumed that a third cell may attach itself by its posterior end to the agglutinated pair. Finally more cells come in and join the same way, eventually giving rise to tangles of hundreds of cells. In these groups or masses the whips are always on the outside or the periphery, whereas in rosettes as described by Novy and MacNeal (1905) the whips are always on the inside. On the fifth day of incubation the agglutination was more pronounced and by the sixth day multiplication had not ceased as the agglutinated masses were bigger.

On the sixth day of incubation, four culture tubes (A, B, C, and D) were incubated at 39°C while tubes E and F were left at 25°C. Twenty-four hours later, all four tubes at 38°C had mostly metacyclic trypanosomes and a few crithidial forms; the two culture tubes that were left at 25°C had nothing but leptomonad stages. All six cultures were quite

healthy as there was no sign of bacterial contamination and the leptomonad forms at 28°C were dividing very rapidly.

By the eighth, ninth and tenth day of culture the tubes at 25°C had reached the peak of growth and there were virtually thousands of leptomonad stages in a single drop of culture medium, all clumped together in masses. Although no quantitative studies were carried out, it seemed that the number of metacyclic trypanosomes in the tubes incubated at 38°C remained relatively the same.

The metacyclic trypanosome on the average is 10 μ long and 3 μ wide at its widest. The kinetoplast, which stains red in Giemsa's Stain is approximately 3.5 μ from the posterior end. The nucleus is near the center of the body. The undulating membrane which does not stain in Giemsa's Stain was studied in live preparations. The free flagellum measures approximately 1.5 μ in dead unstained preparations in saline.

On the tenth day of culture bacterial contamination was seen in tube A, the leptomonad population declined very rapidly and twenty-four hours later there was virtually none left in the culture tube. Culture tube B (39°C) was inoculated into a 'clean' yearling blue grouse. The inoculation was made subcutaneously. Two four-week old domestic chicks were likewise infected with the metacyclic forms from two other culture tubes (C and D). A third domestic chick was injected with all the leptomonad forms from a 25°C culture.

Examination of the experimental birds for the presence of S-shaped trypanosomes in the circulating blood was done twenty-four hours later by direct examination of blood from the brachial vein. All birds were negative for parasites in the first twenty-four hours. S-shaped trypanosomes were detected in the blood of the yearling blue grouse forty-eight hours later by direct blood examination. Blood from all three domestic chicks were still negative seventy-four hours after inoculation of the cultural forms. This was confirmed by the absence of leptomonad forms in cultures made from the blood of the domestic chicks obtained forty-eight and ninety-six hours after the inoculation of the cultural forms into the birds.

Black flies dissected five to six days after they were collected from the exposure grouse had thousands of leptomonad forms in them. These were recovered in the fore gut and in the vicinity of the salivary glands. These are in all probability the dividing forms of the trypanosomes from the exposure grouse. They are not unlike some of the leptomonad forms from the in-vitro culture study (Table X). Manwell (1961) had stated that trypanosomes in cultures assume the stages normally found in the insect vectors. No metacyclic forms were ever detected in dissected flies.

Discussion

The measurements of the S-shaped trypanosomes from the blood of the blue grouse seem to indicate that it is T. gallinarum

TABLE VIII

Summary of Experimental Work on Blue Grouse Trypanosomes

Culture Tube	Temperature Day 0-Day 6	Culture Forms Day 0-Day 6	Temperature Day 6-Day 10	Culture Forms Day 6-Day 10	Bird infected with Day 10 forms	Result of Infection
A	25°C	leptomonads	39°C	metacyclic trypanosomes		
B	25°C	leptomonads	39°C	metacyclic trypanosomes	Yearling Blue Grouse	S-shaped Trypanosomes in circulating blood
C	25°C	leptomonads	39°C	metacyclic trypanosomes	4 week old Domestic Chick	negative
D	25°C	leptomonads	39°C	metacyclic trypanosomes	4 week old Domestic Chick	negative
E	25°C	leptomonads	25°C	leptomonads	4 week old Domestic Chick	negative
F	25°C	leptomonads	25°C	leptomonads		

TABLE IX

Comparison of Blue Grouse Trypanosomes with measurements of Trypanosoma avium and T. gallinarum as published in literature

Trypanosome	Author	Total Length	Width	PK distance	PK/TL	PN distance	PN/TL
T. avium	Baker (1956)	49.6 μ	5.1 μ	12.8 μ	0.26	23.5 μ	0.47
* T. paddae	Wenyon (1910)	-	-	-	0.43	-	0.57
* T. gallinarum	Bruce et al (1911)	52.8 μ	-	11.7 μ	0.22	25 μ	0.47
* T. gallinarum	Wenyon (1910)	-	-	-	0.22	-	0.51
Trypanosoma from Blue Grouse	Woo	53.2 μ	5 μ	12.6 μ	0.24	26.2 μ	0.49

* From Bennett, 1961.

TABLE X

Summary of Measurements and Ratios of Blue Grouse Trypanosomes and those of cultural forms

Trypanosome Form	Source of Specimens	No. measured	Total Length (T.L.)	Width at Nucleus (W)	P.K. distance	PK/TL	P.N. distance	PN/TL	Free Flagellum Length
S-shaped Trypanosome Form	Circulating Blood	19	53.20μ	5μ	12.6μ	0.24	26.2μ	0.49	5-7μ
Metacyclic Trypanosome Form	Culture (39-40°C)	15	10μ	3μ	3.5μ	0.35	4.6μ	0.46	1.5μ
Leptomonad Form	Culture (25°C)	21	8.8μ	1.5μ	5.56	0.63	5.56	0.63	7μ
Leptomonad Form	Simulium	25	10.2μ	2.24μ	6.16	0.61	6.16	0.61	6.5μ

Bruce et al. 1911 (Table IX), but failure to infect young domestic chicks with the metacyclic forms from the in-vitro culture positively negate this identification (Table VIII). Fallis in a personal communication to Dr. J.R. Adams feels that the trypanosomes found in ruffed grouse in Ontario is T. avium. Bennett (1961) had also tentatively identified the trypanosomes from various birds in Algonquin Park, Ontario, as T. avium. His list of naturally infected birds in Algonquin Park included ruffed grouse. The cultural characteristics of the trypanosomes from Vancouver Island blue grouse did not resemble those of T. avium as given by Novy and MacNeal (1905). Novy and MacNeal (1905) indicated that rosettes were quite abundant in T. avium cultures incubated at room temperature (approximately 22°C). No rosettes were ever found in my cultures, there were only agglutinations of the leptomonad stages after the fourth day of incubation at 25°C. However, until more information concerning the life cycle and host specificity are available, this author hesitates to assign the trypanosome to any existing species or to erect a new species to contain it.

The cultures were incubated at 25°C during the first six days because that is the average summer temperature in the field. At this temperature the cultures were dividing at a tremendous rate, but only leptomonad forms were ever detected. Field studies also indicated that only leptomonad stages were present in the flies (Simulium aureum and Cnephia

minus) five to six days after they had fed on the infected grouse. No leptomonad stages were detected in Culicoides during the same period. This does not mean that Culicoides species are not vectors for this trypanosome. As the infection in the grouse was very light, the chance of a Culicoides picking up a trypanosome in the blood meal was at least five to six times less than that of a black fly; and less than 10% of the black flies dissected had leptomonad stages. Bennett (1961) reported that any biting fly can act as vector for T. avium in Algonquin Park. With this study in mind this author feels that the Culicoides spp. are potential vectors. Failure to find trypanosomes in the Culicoides dissected this summer may only indicate that they are not efficient vectors in cases of low infections in the host birds.

In the in-vitro studies all the leptomonad stages changed to the metacyclic trypanosomal forms if the incubation temperature was raised to 39°C for twenty-four hours: in those that were left at 25°C there were only leptomonad stages. The raising of the incubation temperature from 25°C to 39°C would simulate the temperature change affecting the leptomonads if they were inoculated from the fly to the bird. Since all the leptomonads changed to the trypanosomal forms at this higher temperature, it is not too presumptious to postulate that the leptomonads can be the infective stage (that is, it can cause an infection if it were inoculated into a bird). Experimental studies indicate that the mortality rate of flies would

increase very much if the temperature was raised above 30°C for any length of time. This would tend to indicate, that under natural conditions the factor responsible for the change of the leptomonad stage to the metacyclic trypanosome stage in the fly (if this occurs at all) would be a physiological stimulus from the fly rather than an external stimulus such as temperature.

The life cycle of the trypanosome was completed by injecting the cultural metacyclic forms into a 'clean' yearling grouse (Table VIII). Hence it has been proved experimentally that the metacyclic forms from the culture can be the infective form. No leptomonad forms from the culture were ever injected into grouse as there was no 'clean' grouse available. As stated earlier in the discussion the leptomonad forms may be the infective forms in natural transmission as no metacyclic forms were ever found in the flies dissected.

SUMMARY AND CONCLUSIONS

I. Haemoproteus

1. A new species of Haemoproteus was described from the Vancouver Island blue grouse (Dendragapus obscurus fuliginosus Ridgeway). The gametocytes of H.dendragapi are round and the occurrence of this species seems to be localised in the Nanaimo Lakes Area.
2. The rate of gametocyte development of H. canachites in the Vancouver Island blue grouse seems to be much more rapid than that described by Fallis for H. canachites in ruffed grouse in Ontario.
3. Young tissue stages of H. canachites were described from the lung smears of grouse chicks.

II. Leucocytozoon

4. The life cycle of L. bonasae was completed by a new vector (Cnephia minus); also Simulum aureum as a vector for L. bonasae was confirmed. Attempts to infect domestic chicks were unsuccessful.
5. Descriptions of the change in form of L. bonasae gametocytes with age was accomplished by making successive smears from an infected bird.
6. A probable new Leucocytozoon species was described from the Campbell River Area, Vancouver Island.

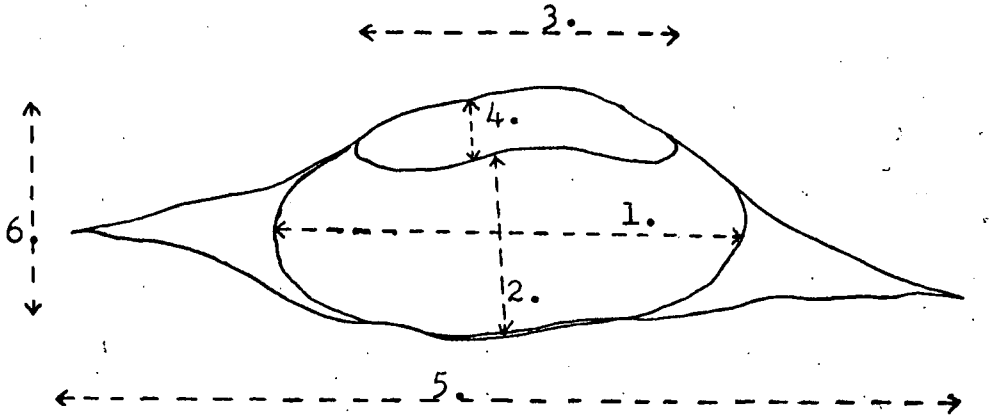
III. Trypanosoma

7. Description of the S-shaped form in the blood of the bird was given.

8. In-vitro culture of the trypanosome was accomplished in a diphasic medium, and description of the fly stages were compared with those of the cultural forms.
9. The life cycle of the trypanosome was completed by injecting the metacyclic forms from the culture into a 'clean' yearling grouse. Attempts to infect domestic chicks were unsuccessful.

This study confirms the work of earlier workers (Fowle 1946; Adams and Bendell 1953; and Bendell 1955) who found that the Vancouver Island blue grouse are quite heavily infected with parasitic blood protozoa of three different genera, and provide a precise characterization of the species which are present.

Figure 1. Key to the Measurements made on Leucocytozoon.



1..... Parasite Length.

2..... Parasite Width.

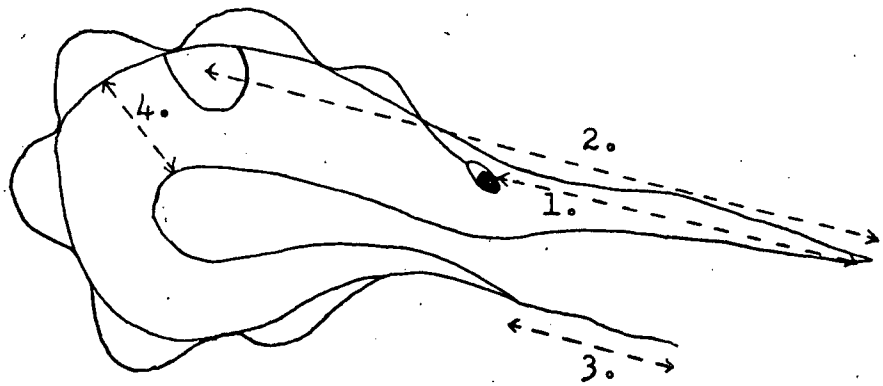
3..... Host Cell Nucleus Length.

4..... Host Cell Nucleus Width.

5..... Parasite-Host Cell Length (from one end of the attenuation to the other.)

6..... Parasite-Host Cell Width (the whole width of the parasite host cell complex.)

Figure 2. Key to Measurements made on Trypanosoma.



1..... Posterior Tip to Kinetoplast.

2..... Posterior Tip to Center of Nucleus.

3..... Length of Free Flagellum.

4..... Width of Parasite (just anterior of nucleus).

T.L.... Total Length of Parasite (length from the posterior tip to the anterior end just before the free flagellum starts).

Figure 3. Photomicrograph of Haemoproteus canachites and H. dendragapi n.sp. Gametocytes.

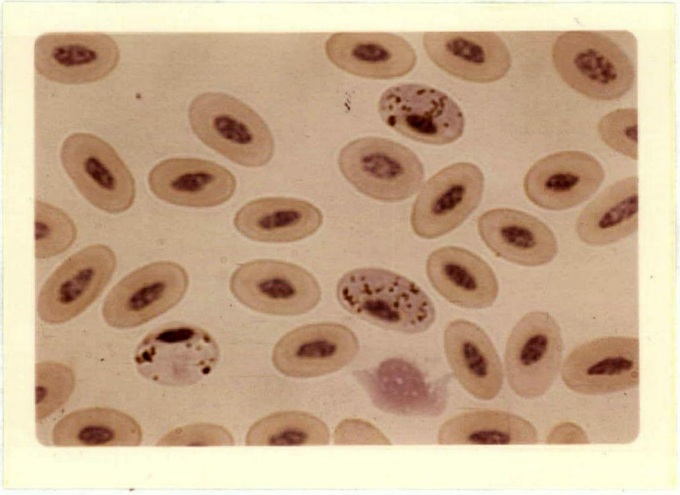


Figure 4. Photomicrograph of Immature Male and Female Haemoproteus dendragapi Gametocytes.

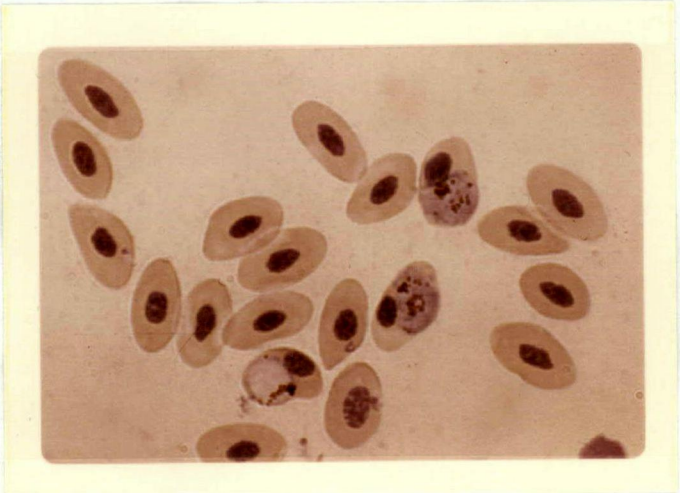


Figure 5. Photomicrograph of Mature Male Haemoproteus dendragapi Gametocytes.

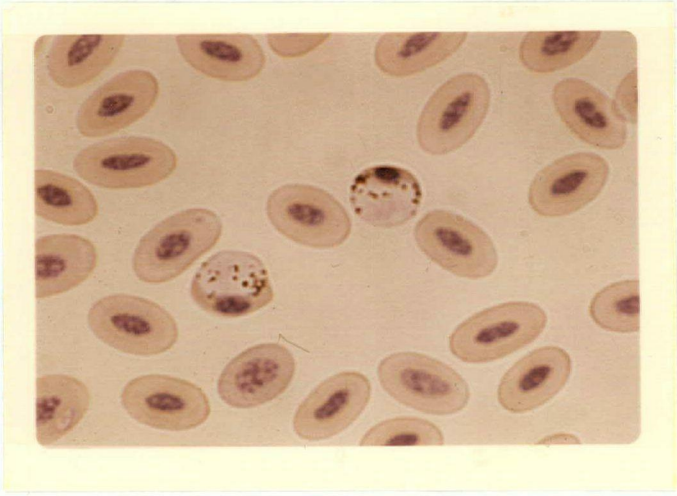


Figure 6. Photomicrograph of Attenuated Form (Type I) of Leucocytozoon bonasae.

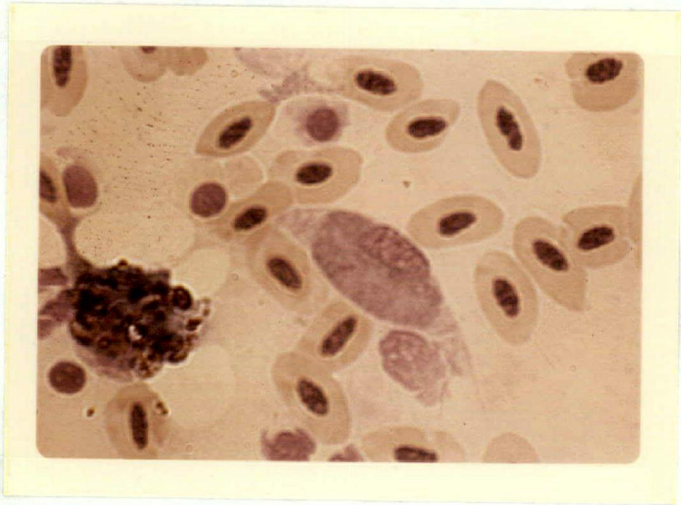


Figure 7. Photomicrograph of Attenuated Form (Type II) of Leucocytozoon bonasae.

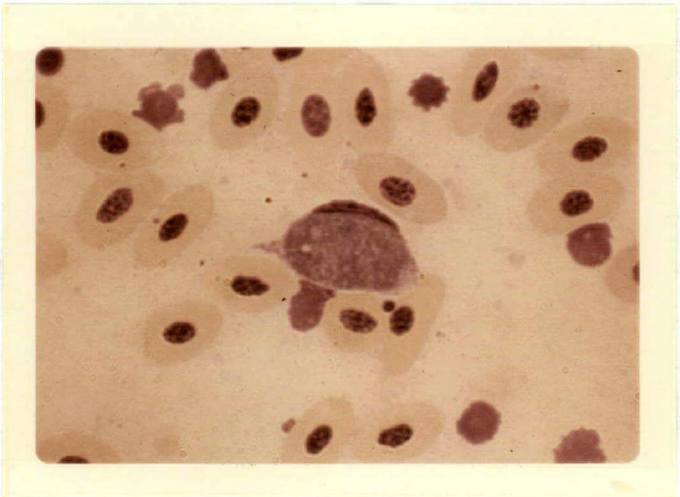


Figure 8. Photomicrograph of Round Female Leucocytozoon bonasae Gametocyte.

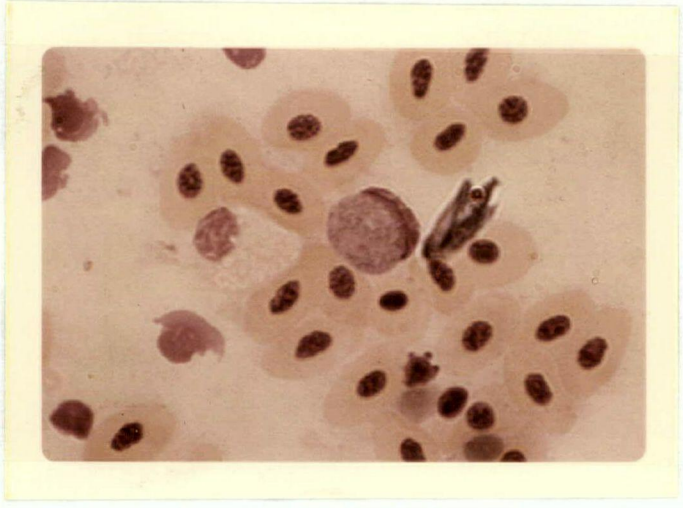


Figure 9. Photomicrograph of Female Leucocytozoon 'A' Gametocyte.

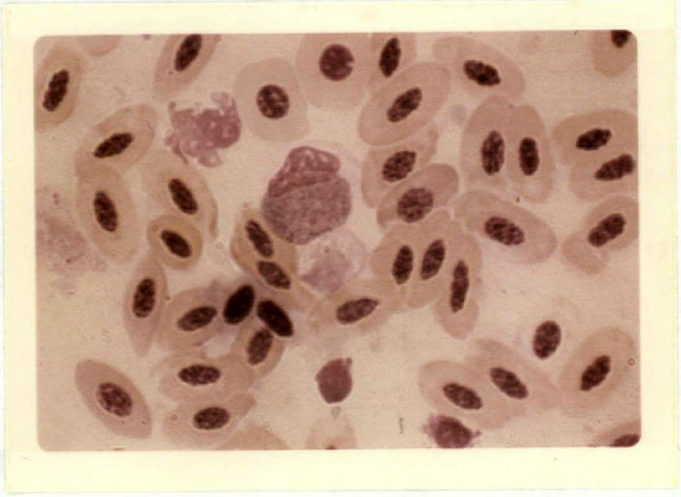


Figure 10. Photomicrograph of Tissue Stages of Haemoproteus canachites.

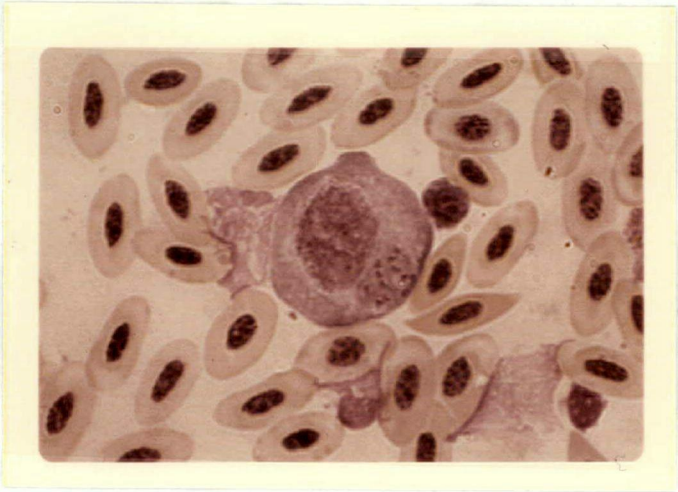
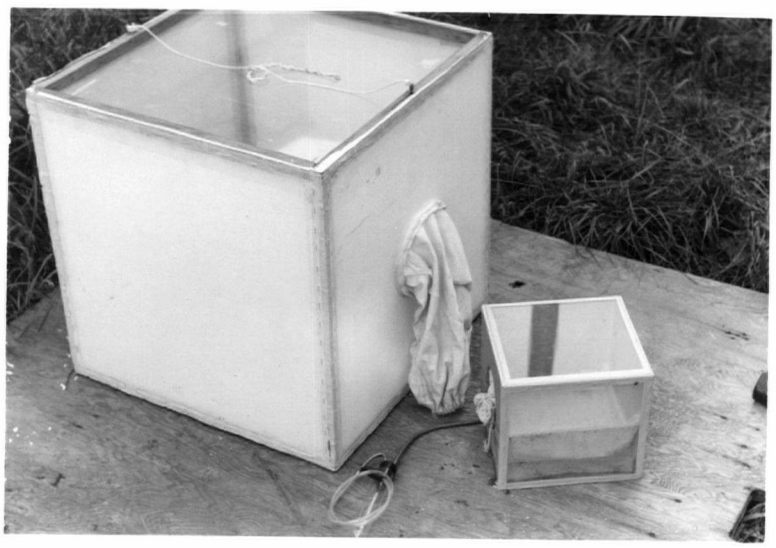


Figure 11. Photograph of Hooded Bird besides Collecting Cage.

Figure 12. Photograph of Field Equipments for the Collection and Maintenance of Engorged Flies.



Figures 13, 14, 15, and 16 are prepared from fixed and stained preparations. All figures are drawn to same scale.

Figure 13. Exflagellation of microgametocyte of L. bonasae in stomach contents of Simulium aureum that ingested gametocytes 1 hour previously.

Figure 14. Zygote of L. bonasae in stomach contents of Cnephia minus that ingested gametocytes 12 hours previously.

Figure 15. Ookinete of L. bonasae in stomach contents of Cnephia minus that ingested gametocytes 12 hours previously.

Figure 16. Sporozoite of L. bonasae recovered from salivary gland of Cnephia minus 10 days after ingesting blood meal.

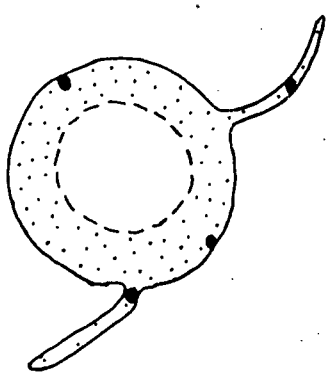


Figure 13.

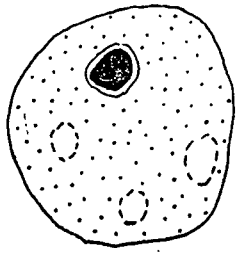


Figure 14.

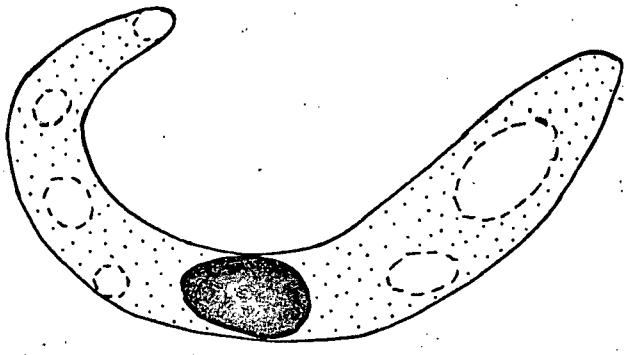


Figure 15.

Figure 16.

5μ

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