GAMETOCYTE PERIODICITY IN *LEUCOCYTOZOON SIMONDI* MATHES AND LEGER INFECTIONS AND ALTERATIONS NOTED AFTER PASSIVE IMMUNIZATION

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INTRODUCTION

Since its discovery in 1890 by Danilewsky, *Leucocytozoon* has remained an incompletely understood protozoan parasite. O'Roke's early description (1934) of the life cycle of *Leucocytozoon simondi* Mathes and Leger was relatively accurate for the sexual cycle in the definitive host, the vector. His description of the asexual cycle in the duck has been proved inaccurate. The literature today has numerous descriptions of the asexual cycle, but due to the conflict among them, the exact cycle is still poorly understood.

The work being reported here is an attempt to more accurately describe the cycle of *L. simondi* in the domestic duck, *Anas platyrhynchos* Linneaus, and to determine some of the defense mechanisms of the host. Standardization of techniques employed for the exposure to vectors and determination of parasite density was attempted. An attempt is also made to explain some of the observed phenomena of the infection in terms of immunity.

There are both economic and academic reasons for studying this organism, although one can not always separate the two. Both duck ranchers and sportsmen are interested in the control of *L. simondi*. Hatcheries can be located in nonendemic areas to prevent transmission, but the sportsman is concerned because the organism is pathogenic to both ducks and geese of many species in their breeding grounds. At the time of nesting, the adult birds relapse if they have been previously infected, making the parasite readily accessible to the vector and subsequently to the young birds who are extremely susceptible.

Today taxonomists relate *Leucocytozoon* to the genus *Plasmodium*. If the relationship is correct this organism affords an excellent form with

which to study methods of immunization and treatment which might aid in the problem of human malaria eradication. This problem has been under considerable study ever since malaria control was accomplished a number of years ago. The line of attack under investigation here is the host's defense mechanisms. This has been complicated in Plasmodium research by the occurrence of gametocytes and schizonts in the host's erythrocytes. Leucocytozoon, however, affords a better opportunity to study each stage separately with respect to immunity since only the gametocytes occur in the peripheral circulation while the asexual schizonts occur in the internal organs.

LITERATURE

Leucocytozoon simondi infections are described in textbooks as the cause of a blood protozoan disease of waterfowl, initially acute with a gradually decreasing parasitemia developing into a chronic infection which relapses regularly each spring during the breeding season. Transmission is biological, through various species of *Simulium* and possibly *Culicoides* (Fallis and Bennett, 1961). The vector is also the definitive host with the parasite exhibiting developmental stages very similar to those of *Plasmodium* in the mosquito. Transmission occurs in the northern breeding grounds of the ducks and geese which overlaps the endemic area of the black fly. Since no transovarial transmission has been reported to occur in the vector, the flies are believed to become infected from relapsing parent birds who have recovered from previous *Leucocytozoon* infections and have returned to the breeding grounds. The organism is then transmitted to the newly hatched ducklings and goslings.

The course of the infection in the vertebrate host is vaguely outlined in most texts. It is described as having a prepatent period of 6 to 14 days after which time the gametocytes appear in the blood. The gametocytes reach their maximum number by the eighth to tenth day, and if death does not occur the gametocyte number drops to near zero by the thirtieth day and remains at a low level or latent condition until the following spring. This latent condition or lack of total recovery is believed to be the result of multiplication of the tissue stages of this organism. These stages become apparent, upon autopsy of the infected bird, in the liver, spleen, lungs, brain and most organs with lymphatic tissue. Tissue stages are present prior to the appearance of the gametocytes in the blood and are present, as far as is known, for the

remainder of the life of the host.

Morphologically, stages in the vertebrate host are described as having two distinct gametocyte types and two distinct schizont types. The predominant gametocyte type is reported to be round, but dimensions are seldom stated. The other type of gametocyte reported is the elongate form. This measures 14 to 22u long. In either case the microgametocytes are slightly smaller than the macrogametocytes. The gametocytes are contained within a host cell which has not as yet been definitely identified. It is believed to be either a cell of the lymphocytic series or an erythrocyte in a greatly distorted condition (Figure 2). The tissue stages commonly reported are the liver form or "hepatic schizont", measuring 11 by 18u and the lymphoid or "megaloschizont", measuring 60 by 164u. Both of these forms are in the organ cells and are never seen in the peripheral blood.

Description of the symptoms and pathogenicity are essentially in accord with one another. The course of infections is consistent regardless of the sex of the infected bird. The most susceptible birds are the newly hatched, under the age of four weeks. An outbreak of Leucocytozoon infections in ducks is characterized by its suddenness of onset, listlessness, rapid breathing, and lack of interest in feed. Death occurs by the 10th or 11th day of infection if recovery is not complete.

Fallis et al. (1951) and O'Roke (1934) have clearly shown that Leucocytozoon is transmitted to the duck by means of sporozoites which have developed in the black fly.

After introduction of the sporozoites into the duck by black flies, there occurs a prepatent period of approximately 6 to 14 days, depending on the method of exposure used by the investigator. Fallis et al. (1951)

stated that a greater cyclic synchrony of parasitemia and patency occurred among birds injected with macerated black flies than occurred with natural exposures. Their periods of exposure varied from one to several days. They also suggested that there is probably one asexual generation in the internal organs of the duck prior to gametocyte patency, and that this cycle is not necessarily synchronous among all schizonts and that the number of sporozoites introduced may affect the prepatent period. In a later work Fallis et al. (1956) reported the finding of Dr. Ritchie of the Department of Pathology, University of Toronto, in which he found hepatic schizonts in the Kupffer cells of the liver on the second day of the infection. From this observation Fallis et al. reconsidered their first suggestion and postulated two possible asexual cycles prior to gametocyte patency--the first generation in the Kupffer cells producing the second generation which develops in the spleen and other lymphoid tissue where they become megaloschizonts. These then produce the gametocytes which appear in the circulation. These findings correlate nicely with their observations of 1951 in which they noted that the maturation time of a gametocyte is approximately 48 hours or less.

Cowan (1955), using birds exposed for five days for a study of the tissue schizonts, concluded that the megaloschizont is the final prepatent generation resulting from some previous asexual cycle, and is the sole contributor of gametocytes to the peripheral blood. This is in agreement with the views of Fallis et al. (1956).

Huff (1942) put forth three possible explanations for the occurrence of two types of schizonts: 1) hepatic schizonts may represent a first generation followed by megaloschizonts, 2) the difference may be a manifestation of the host tissue involved and 3) the two forms may

represent stages of different parasites. He believed the first explanation to be the most probable.

The gametocytes appear in the blood approximately seven days post exposure, marking the start of patency. Patency is immediately preceded by an increase in the leucocyte count (Fallis et al., 1951). The exact time of gametocyte appearance varies somewhat from author to author and seems to depend on the duration of exposure to the vectors. Chernin (1952c) reported that prepatency ended with the appearance of immature stages or trophozoites in the blood. He described these as the very small stages and the large round stage. Chernin also reported that only during the first two days of patency are multiple infections noted. Similar superinfections are also reported by Cook (1954). No reports of multiply infected host cells past this time have been found.

Hartman (1929) claimed that the earliest forms seen in the circulation occurred in erythrocytes. Cook (1954) and Ramisz (1962) agree with this view. Cook states that lightly infected birds had parasitized erythrocytes only. In heavier infections she reports that lymphocytes were also noted to be infected but that erythrocytes outnumbered the lymphocytes as carriers of the parasites. She differentiated erythrocytes from lymphocytes by using benzidine-peroxide as an indicator of hemoglobin. She also reported finding two-thirds of the erythrocyte infecting trophozoites to be in reticulocytes. With the same indicator she also found hemoglobin in the host cells of the large round gametocytes, but no hemoglobin was found in the elongate host cells. This she attributes to the assumption that the hemoglobin has been altered or lost as the parasitized cell grows, thereby losing the ability to stain.

Huff (1942) recalled Mathes and Leger's proposal that the rounded

forms were invaders of mononuclear leucocytes while pyriform or elongate parasites were in erythrocytes or erythroblasts. Huff's own work indicates that the smallest stages occurred in myelocytes, late polychromatophil erythrocytes, lymphocytes, monocytes and macrophages. There was no evidence, however, that any form developed past the earliest stages in cells other than monocytes or macrophages. He also reported finding growth stage series only in lymphocytic cells and concluded that erythrocytic infections never developed to maturity.

Fallis et al. (1951) found what they believed to be erythrocytes and cells of the lymphocytic series to be infected. They also concluded that there was possible lymphocytic involvement since blood passage would sometimes transmit the infection. Injections of heart, lung, liver and spleen also produced infections. They considered lymphoid cells as carriers of infective stages which later lodged in some tissue where further development occurred.

Savage and Isa (1959) concluded that although small lymphocytes could be parasitized, they presented a physical impossibility to becoming gametocyte carriers, whereas Huff (1963) proposed the rapid growth of small lymphocytes after invasion. They postulated that monocytes or macrophages were the most probable carriers of gametocytes on the basis of: 1) volume of the cell, 2) the hyperparasitism known to occur in these cells, and 3) a residual differential count of white cells.

Fallis et al. (1951) observed that ducks exposed for a single day showed predominantly small and large round forms of the gametocyte with elongate forms appearing later in the infection. However, birds which they exposed to continuous infection showed more numerable small and large round forms. In comparison, Chernin (1952c) showed that one day

exposures produced a consistent appearance of elongate gametocytes on the fifth day after patency while eight day exposures showed a great deal of variation as to when elongate forms appeared. The range was from the first to the twelfth day of patency. Although he did not interpret his data in this way it can clearly be seen from his table (Table VII). He also reported that there was a decline in immature forms on day four of patency and suggested that this reflected a halt of schizogony. Seven days later rapid clearance of the gametocytes was noted. This would correspond with the findings of Fallis et al. (1951) in which they noted that the normal life span of a gametocyte was about six days in a recipient host.

Cowan (1955) states that during the first several days of patency merozoites were released into the host's tissue upon rupture of the schizont wall, and due to the hyperemia associated with the infection the merozoites would be in direct contact with blood. Fallis et al. (1956) observed that the maximum number of megaloschizonts in the spleen occurred 7 to 12 days post exposure (day 1 to 5 of patency). No exposure duration was mentioned. This agreed with his earlier assumption that there were more numerous megaloschizonts in birds which may have gone through more than one schizogony, and that a complete schizogony may take only 2 to 4 days.

Several workers have reported changes in the number of schizonts in infected organs with respect to the time of the infection. Cowan (1955, 1957) noted that on the first and second day of patency every organ was infected. On the third day only the heart and brain had visible schizonts and by the fourth day the brain alone showed schizonts. He noted that schizonts failed to mature in the brain and assumed that

this was an unsuitable site for development. Fallis et al. (1951), however, reported that from day four to day nine post-infection, the spleen continued to show megaloschizonts. On day ten to eleven the spleen, lung, brain and lymphoid tissue all contained these schizonts.

Upon reaching maturity the megaloschizonts undergo a process of septa formation as described by Cowan (1955). This occurred only after maximum schizont size was attained but before internal differentiation ceased. He noted that the septate schizonts usually have the same type of cytomere in each compartment but that occasionally one occurred in which the compartments had cytomeres in various stages of development. He is also of the opinion that maximum growth of the schizont is limited by encapsulation or by limits of the tissue elasticity. This limit on the cell, he concluded, produces a pressure on the central body by the proliferating cytomeres and internal fluids of the schizont, causing rupture of the schizont and the release of the merozoites. This interpretation of the mechanism of schizont rupture is a result of his belief that the central body is a primordium from which primary cytomeres are cut. These then produce secondary cytomeres and so on until the schizont is fully mature. This is in direct opposition to the belief of Huff (1942) and Wingstrand (1947) who believe that the central body is the remnant of an enlarged and distorted host cell nucleus.

Agreement among workers as to the exact length of prepatency is not consistent due to the various durations of exposure time. Their views on the course of the infection once patency has begun are more vague than in disagreement. Fallis et al. (1956) reported that maximum spleen size corresponds with the peak of gametocyte density as determined by counting gametocytes per unit time. This peak occurred between the

tenth and fourteenth days post-exposure (3 to 7 days into latency). Chernin (1952c) felt that the gametocyte peak was between the third and ninth day post infection. The two authors used exposure times varying between 1 and 8 days. There is agreement among several authors, however, that the parasite numbers rapidly reach a peak and then gradually decrease (Fallis et al., 1951, 1956; Huff, 1942; and O'Roke, 1934). Chernin and Sadun (1949) expressed the possibility that a fluctuation in gametocyte levels occurred during the first thirty days of the primary infection. This was based on counting parasites per microscopic field. Chernin (1952c) later discounted his belief and conformed to the opinion of others. At this time he also attempted to correlate the presence of round and elongate gametocytes in the host. His definition of immature gametocyte was equated with the large round forms as well as the small trophozoites. Since this was later proven to be an incorrect assumption by Fallis et al. (1951) and Rawley (1953) the forms will be referred to here as round and elongate since it is difficult to tell exactly how many trophozoites he counted. Chernin's table of peak densities of round and elongate forms are reproduced in Table VII. Chernin interpreted the two curves to indicate that no correlation between round forms to elongate forms could be made. Fallis et al. (1951) using one minute counting periods show a graph of a "typical" parasitemia (Figure 11). It is interesting to note the three peaks shown on the graph. Fallis refers to these peaks as being regular in occurrence but claims that the gametocyte cycle is completely asynchronous or irregular.

Briggs (1960) reported that his studies supported the views of Chernin; that there is no correlation between round and elongate

gametocytes in individual ducklings. Martin (1932) claimed that the elongate forms could not take part in gametogenesis and therefore the round forms were the only functional form. He postulated that the elongate gametocytes were decadent individuals or "overmature".

The work of Fallis et al. (1951) and Rawley (1953) shows that both round and elongate forms of gametocytes can exflagellate. This invalidates Chernin's supposition that the round forms are immature.

Three possibilities were put forth by Chernin (1952c) as to what caused the pleomorphic gametocyte forms: 1) fundamental host differences, 2) manifestations of specific immune responses and 3) strain differences in the parasite itself. Other commonly suggested reasons are the type of host cell invaded (Fallis et al., 1951) and two species of parasites (Cook, 1954; Fallis et al., 1951). Cook points out that if host cell invasion made the difference then both round and elongate forms should appear at the same time due to their simultaneous release from the schizonts (assuming that there was a simultaneous release). She also notes that although Chernin (1952c) observed four birds which showed no elongate forms during the entire course of the primary parasitemia, that no cases of elongate forms only have been reported for L. simondi. This would be highly improbable if two species of parasites were involved. Fallis et al. (1951) suggested that this phenomenon might be due to the inability of the host cell to change shape when infected with a large parasite, while Huff (1963) suggested that lymphocytes may be stimulated to rapid development after invasion. In looking for a relationship between the two forms, Rawley (1953) reported finding no transitional stages between the two, though she did see young stages in what appeared to her to be elongate host cells. She also reported seeing elongate

forms "round up" while she observed them alive, and seeing round forms become elongate just prior to exflagellation; but not in all cases of exflagellation did this occur. Briggs (1960) compared the parasitemias in the Pekin with those in Muscovy ducks in an attempt to elucidate the confusion concerning the two forms of gametocytes. He noted that the average of the two forms in the Pekin was about equal, while Muscovies seldom showed as many as 5% elongate forms. His graphs resemble very closely those of Chernin for the appearance and density of the gametocytes in the Pekin.

The spring relapse which occurs in birds with latent infections is also not fully explained. Huff (1942) postulated that the increased number of gametocytes in the peripheral blood following a low winter parasitemia was due to a true relapse since no vectors were available. Birds which are held in an endemic black fly area showed the same blood picture as did those kept in an area of no transmission (Barrow, unpublished). Huff's results showed that young stages of Leucocytozoon and Haemoproteus became apparent in April. In accord and in contrast to this, Chernin (1952b) reported the same occurrence of gametocyte increase in the spring of the year but that immature forms did not become evident until one month after the relapse, whereas they constituted 20% of the gametocyte population during the winter months. His reference to immature forms includes the round forms. From his data he concluded that schizogony in the tissues probably occurs throughout the winter months, which explains the immature gametocyte population during that time.

Due to the correaltion among relapse, increased day length, and egg production or breeding, Chernin (1952b) carried out experiments which were intended to clarify the relationship among the three

occurrences. He found that if the light on the birds was increased from 10 hours to 16 hours between November and December, that egg laying and relapse occurred in January. Eleven to 60 days post-egg-production the parasitemias increased 8 fold as compared to pre- egg-production. All relapses lasted not less than three months. Interestingly enough, males also relapsed during the egg laying period. Relapses occurred among females isolated from males, males isolated from females and males and females together during this period.

It has been pointed out by Clark (1964) that a species of Leucocytozoon in the Magpie showed gametocytes in the tissues throughout the year, even when the blood was negative for them.

Briggs (1960) reported that anemia, due to the Leucocytozoon infection, was an important factor or the primary cause of death in the Pekin. He also noted that severely infected birds died before the appearance of elongate gametocytes and before the peak of round forms. Cook (1954) relates anemia to destruction of erythrocytes by the invading parasites. Huff (1963) suggested the possibility that autoimmune responses similar to those reported for malaria may account for the anemia.

Chernin (1952a) made several observations on mortality as a result of L. simondi infections. He remarked that the greatest losses were among ducks less than four weeks old, and that indigenous birds suffered much lower losses than did imported birds exposed for brief periods (8 days). The highest mortality occurred between 10 and 19 days post exposure, with a gradually decreasing number of deaths occurring until latency.

Premunition is a commonly reported phenomenon among organisms which

leave a residual population after the host has reduced the parasite numbers and symptoms of the disease have disappeared. This results in the lifetime immunity of the host. Malaria is reported to produce just such a response, and Leucocytozoon, supposedly related to Plasmodium, would be expected to produce such a lasting immunity in the host.

It is not uncommon to see reports of birds showing relapses for a number of years after their first exposure to the organism, which indicates that the organism is still present and being kept in check by the host. Indeed, it has been reported by several authors (Fallis et al., 1951; Chernin, 1952c) that recovered birds do exhibit premunition. There is, however, an interesting finding by some authors concerning this phenomenon. Fallis et al. (1951) reported that recovered ducks exposed one year later succumbed to the second exposure, supposedly due to a lack of immunity. Further experiments showed that birds exposed constantly throughout the summer became immune to reinfection the following year. This work is in agreement with the supposition of Hartman (1929). He reported that evidence pointed to little resistance as a result of low infections.

Chernin (1952c) postulated that immunity probably developed very rapidly during primary infection, evidenced by the steady decrease in number of gametocytes after the initial peak.

Fallis et al. (1951) reported that there was no difference between splenectomized and whole ducks which were exposed for five days. They did, however, note that there was a higher level of parasitemia and longer duration of parasites before latency, in birds splenectomized after exposure. He concluded that the spleen must be an important defense organ and that this might account for the larger number of

schizonts seen in the spleen after the initial cycles.

Briggs (1960) reported that in addition to the low percentages of elongate forms seen in the Muscovy ducks that they had much lower parasitemias and died later, if at all, than did the Pekins. He attributed this to a natural resistance in the Muscovies.

Cowan (1957) did an extensive histological study on the host's reaction against the megaloschizonts. He noted five visable host reactions: 1) encapsulation or possibly walling off by the host, 2) phagocytosis, 3) necrosis, 4) phagocytosis and necrosis, and 5) destruction by inflammatory cells. These reactions were noted only in cells which had reached their maximum dimensions. Heterophils and macrophages were the most active phagocytic components, but in cases where only a few host cells were present they were heterophils and occurred near the outer margin of the schizont. He noted that as parasite removal proceeded there was an increase in the number of macrophages. There were, however, only inflammatory cells observed in the forms found in the brain. These forms, he noted, did not reach maturity regardless of whether or not there was a reaction by the host. There were some schizonts which exhibited both phagocytosis and necrosis at the same time. Others showed necrosis without phagocytosis, which in some cases extended inward from the limiting membrane of the parasite and could be interpreted to indicate that humoral immunity of some sort exists. Which of these mechanisms occurred first, necrosis or phagocytosis, could not be determined by him. He does, however, postulate on the findings of other workers concerning premunition. He believes that constant reinfection keeps cellular elements at a high level thereby conferring a high degree of resistance, while one exposure with subsequent recovery does not

stimulate high cellular activity.

The role of gamma globulin in malarial infections has just recently come to the fore. Work by Coggeshall and Kumm (1937) demonstrated that gamma globulin from recovered monkeys conferred a partial resistance to newly infected animals of the same species providing that the infection was of low intensity. They also demonstrated that there is a species specificity of this globulin by attempting immunization of monkeys infected with Plasmodium inui with globulin from monkeys recovered from P. knowlesi. Monkeys infected with P. knowlesi showed an immunity upon being transfused with the recovered globulin while those infected with the milder P. inui showed no immunity when treated with the same globulin.

Cohen and McGregor (1963) reported that the antibodies responsible for humoral immunity to malaria are in the 7S fraction of the gamma globulin. Globulin from uninfected patients showed no activity against malarial infections.

Working with P. falciparum infections in Gambian, African children, Cohen and McGregor (1963) showed that gamma globulin from hyperimmune adults drastically reduced the number of circulating parasites in newly acquired infections, providing the treatment was begun when the earliest stages of the parasite were observed in the blood. There was no immediate reduction in numbers but following the second schizogony after treatment began, the parasite density fell to below 1% of its original value. Along with the reduction of parasite numbers there was an alleviation of all symptoms except pyrexia. This continued even after apparent recovery which indicates that some mechanism other than the parasite's presence in the peripheral circulation causes the elevated

temperature. The protection afforded by this globulin, being passive in nature, lasted only up to twelve weeks, after which time the patient was again fully susceptible to further infection.

These authors suggested that the action of the globulin was against the mature intracellular parasites or on the newly released merozoites. The latter appeared to be the most probable and helped to explain the apparent low level of protection of the malaria antibody, since it had access to the merozoites for only limited times and for short duration. The authors were not able to explain the few remaining parasites which persisted after the initial rapid clearance. It would seem that if the immune globulin caused the clearance of several million parasites that it would also remove the remaining few. Their work also contradicted the belief that it was necessary for the reticulo-endothelial system to be stimulated for a prolonged period before clearance was accomplished. This was noted when infant's blood was rapidly cleared of parasites during an initial infection when treated with immune globulin.

MATERIALS AND METHODS

Set I: Early Summer Exposures

For the pilot experiment, blood was collected from a single one-year-old mallard, Anas platyrynchos, who had recovered from Leucocytozoon infection, at a rate of 30 ml per week until 90 ml of serum was obtained. The serum was then mixed with saturated $(NH_4)_2SO_4$ solution equal to one-half the serum volume, giving a 33% saturated mixture, which precipitated gamma globulin. The precipitate was then redissolved in 0.85% NaCl and the pH adjusted to 7.0. The entire procedure was repeated three times to increase the purity of the product (Figure 1). This procedure is described in Campbell et al. (1963). Globulin precipitated in this manner is reported to contain both 7S and 19S gamma globulin. No attempt was made to separate these further.

The precipitate from the third $(NH_4)_2SO_4$ treatment was dissolved in borate buffered saline, pH 8.4, and dialysed against the same at $4^{\circ}C$ until sulfate ions could no longer be detected upon treatment of the dialysate with $BaCl_2$. The product was then treated with 10 mg/ml penicillin-streptomycin and stored at $-20^{\circ}C$ for four weeks.

Thirty, two-week-old Pekin ducks were exposed to the vectors of Leucocytozoon simondi on June 16, 1964, at Indian River, Michigan. This is approximately thirty miles south of the Straits of Mackinac, and a known transmission area for Leucocytozoon. The exposure time was twenty-four hours in the endemic area. This timing was to insure a twenty-four hour or less difference in parasite development. On June 17, twenty-four hours after exposure, all birds were returned by car to the W.K. Kellogg Bird Sanctuary in Battle Creek, Michigan, an area where transmission of Leucocytozoon to waterfowl does not occur. They were

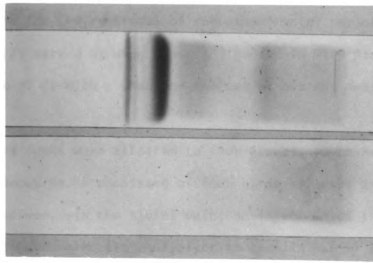

Figure 1. Cellulose Acetate strip electrophoresis of whole pooled duck serum compared to purified gamma globulin from the same source.

Figure 2. A round and an elongate gametocyte of Leucocytozoon simondi in the blood of an infected duck. (Giemsa).

maintained there for the remainder of the experimental period. All birds were individually marked by wing bands and housed in wire pens of approximately 10 ft x 50 ft with a shed for shelter at one end measuring 10 ft x 10 ft.

The exposed ducks were allotted to four groups, to be sampled every 2 to 3 days. Group No. 1 consisted of four birds selected prior to exposure, who received, via the tibial vein, an injection of the above described purified gamma globulin equivalent to that in 5.5 ml of whole recovered duck serum. Group No. 2, also four birds, was given identical treatment as Group No. 1 on the first day of patency based on the first appearance of blood forms. This occurred on June 30th, fourteen days post-exposure. Group No. 3, four untreated birds served as infected controls. Group No. 4 consisted of three unexposed birds who served as uninfected controls. All birds were controls to check transmission of other vector borne parasites which might complicate the course of the organism under study.

Examinations were made every 2 to 3 days between 7:00 and 10:00 P.M. Hemocytological procedures consisted of red blood cell counts made on an A.O. Spencer "Bright-Line" hemocytometer. Blood samples for counts were collected from a puncture wound made in a web vein with a sharpened dissecting probe. The first drop to appear above the surface of the web was touched to a clean microscope slide, smeared, air dried, fixed in methanol and later stained with Giemsa stain. The second drop of blood was drawn up into a red blood cell diluting pipette and diluted with standard Hayem's diluting fluid. As each sample was collected the pipette was placed on a pipette shaker. When samples from six birds were taken, they were counted before the next birds were sampled. This was done to insure a

standard elapsed time between the first and last birds sampled and the time when the red cells were counted. Gametocyte density was calculated from the red blood cell counts and the parasite counts from the stained slides.

All blood samples collected for future serum extraction were drawn from the jugular vein into a 1 ml tuberculin syringe. The samples were then placed in a water bath at 37°C for one hour, after which time they were placed in the refrigerator at 4°C overnight to retract the clot. The following morning the sera were poured off the clots and stored at 4°C for future use.

Set II: Midsummer Exposures

In an attempt to duplicate and clarify the results of the pilot experiment, a second set of young ducks was taken to Indian River on July 16, 1964. This set consisted of thirty-two birds who were exposed for twenty-four hours. At the end of that time they were returned to the Kellogg Bird Sanctuary and housed in a manner identical to that described for Set I. The birds were two-weeks-old at the time of exposure, except two adult birds from the clean controls of Set I.

Five birds were designated as infected controls and kept in the pen with the rest of the experimental birds. Another group of five birds was also kept as controls but was maintained in a 5 ft x 3 ft x 3 ft cage entirely covered by canvas except for a six inch strip along the bottom of each side. This allowed for a normal day-night photoperiod but excluded any direct sunlight. These will be referred to as dark controls. Group No. 1 corresponded to Group No. 1 of Set I. This group consisted of 10 birds, each of which received gamma globulin

equivalent to that in 5 ml of Pekin serum recently recovered from Leucocytozoon. This was administered via the tibial vein immediately prior to exposure to the vectors. Group No. 2 corresponded to Group No. 2 of Set I and consisted of 10 birds. On July 24, eight days post exposure, the first day blood forms were evident, these birds were given the same globulin treatment as was Group No. 1.

The globulin used on birds in this set was obtained from the untreated birds from Set I who had recovered from Leucocytozoon. All serum was pooled, and gamma globulin was prepared as described above. Group No. 1 of this set received globulin from serum collected on July 10, five days after the final gametocyte crisis; and Group No. 2 received globulin from serum collected from the same birds on July 20, fifteen days after the last crisis. This was done because of the limited number of recovered ducks available and the tremendous volume of serum needed to immunize twenty ducklings.

Blood collection and sampling was carried out in exactly the same way as was described earlier for Set I. The number of parasites per mm^3 was also determined in the same way.

Determination of the per cent of immature, mature and elongate gametocytes in the peripheral blood was determined. Classification was based on the size and shape of the parasite. The elongate forms were obvious in most cases, but all forms which showed a pyriform host cell were considered elongate. Round forms included all mature microgametocytes and macrogametocytes with a round or polygonal shape. The immature gametocytes were only those which were smaller than the differentiated gametocytes. This classification differs from that of some other investigators. Figures 2 and 3 show those forms included in the classification.

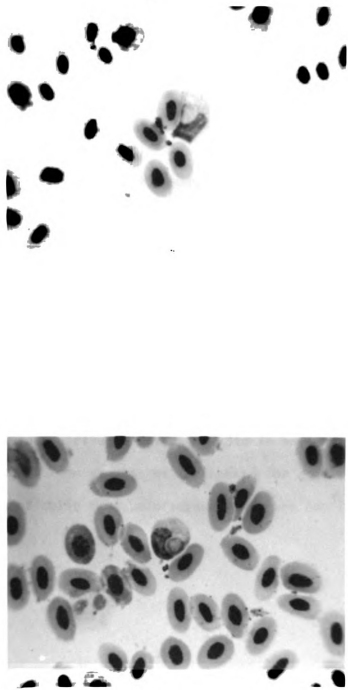

Figure 3. Two immature gametocytes of Leucocytozoon simondi within unidentified blood cells of a duck. (Giemsa).

One hundred parasites were counted and the per cent of each type was calculated. In low level parasitemias, 0.3 to 0.7 per 10^3 red cells, only fifty parasites were counted and in extremely low infections, less than 0.3 per 10^3 cells, only twenty-five parasites were counted.

Two of the birds exposed with this set were the full-grown unexposed controls from Set I. Blood samples were taken at random intervals and the Coombs anti-globulin test performed on their washed red cells. The procedure consisted of washing the cells of the infected ducks four times with 0.85% NaCl and treating with 0.5 ml of a 1% suspension of these cells with rabbit anti-duck globulin serum. This serum was inactivated at 56°C for 30 minutes and adsorbed with normal duck erythrocytes prior to its use in the test. After the cells and anti-serum were mixed they were placed in a water bath at 37°C for thirty minutes. At the end of this time they were centrifuged for one minute in an International Clinical Centrifuge and the button at the bottom checked for agglutination. Controls consisting of cells from uninfected ducks were run in the same manner.

RESULTS

Set I: Early Summer Exposures

Thirty-five per cent of the thirty birds exposed on June 16th became infected as determined by blood examination. The prepatent period, judged by gametocyte appearance, lasted from the 16th to the 30th of June, a total of 14 days. Patency lasted 10 days; until on July 9th only rarely could blood forms be seen.

A single peak in gametocyte numbers was noted between July 2nd and July 5th as determined by calculating the number of parasites per mm^3 of blood.

In the control group which showed 3 out of 4 infected birds, the average number of parasites per mm^3 of blood at the peak count was 5,152 with an accompanying erythrocyte count of 1.3×10^6 per mm^3. This is a loss of approximately 7×10^5 cells per mm^3 when compared to the normal controls who showed about 2.1×10^6 per mm^3. Group No. 1, which received globulin prior to exposure, showed only 2 out of 4 birds infected. In these a parasite density of 3,400 per mm^3 was noted and a blood count of 1.8×10^6 per mm^3, 2×10^5 red cells less per mm^3 than that of normal birds. Group No. 2, the post-exposure treated birds, showed only one infection out of four birds. This bird had less than 1,000 parasites per mm^3 of blood and a red cell count of 2.15×10^6 per mm^3, normal for a healthy duck.

Set II: Midsummer Exposures

Twenty-two of the 32 birds exposed on July 16th, a total of 69%, demonstrated an infection with a prepatency of 7 days. Gametocyte patency lasted until August 15th, a period of 21 days. During this time

calculations showed a fluctuation of parasite density which had at least two peaks and in 11 birds three peaks were evident. These occurred on the 4th, 8th to 10th and 14th to 16th days of patency. Two birds showed some variation to this cycle and will be discussed below.

The controls held in the dark showed identical fluctuations and similar parasite densities as compared to the controls who were kept outside and used as a base line for comparison of all experimental birds. Figures 4a and 4b and Tables I and II show gametocyte counts for the controls and dark controls as an average of each and as individuals in the table. Due to their obvious similarity they will both be considered one control group when comparisons are made.

Eight of the 10 birds exposed in Group No. 1 showed an infection. Of these, four had fluctuations in parasite densities similar to the controls. The other four showed only one peak, which corresponded to the first peak of the controls. Of these four, three had parasite densities of approximately 1,500 per mm^3. The fourth showed a density of 2,250 per mm^3. All but one bird in this group had less acute density peaks than did the controls (Figure 5 and Tables III and IV).

Group No. 2 consisted of 7 infected birds out of 10 exposed. Six of these had parasite densities and blood counts comparable to the controls. #2665 exhibited a single peak that appeared to be a blending of the first and second peaks of the controls (Figure 6 and Table V).

By August 15th all of the birds were recovered or showed extremely low parasite densities.

All birds maintained a normal red cell count of 1.9×10^6 to 2.2×10^6 per mm^3 during the prepatent period. On the first day of patency several of the infected birds showed a slight drop in count.

Figure 4a. Summary graph of Table I showing the mean gametocyte densities per mm^3 blood of the control birds.

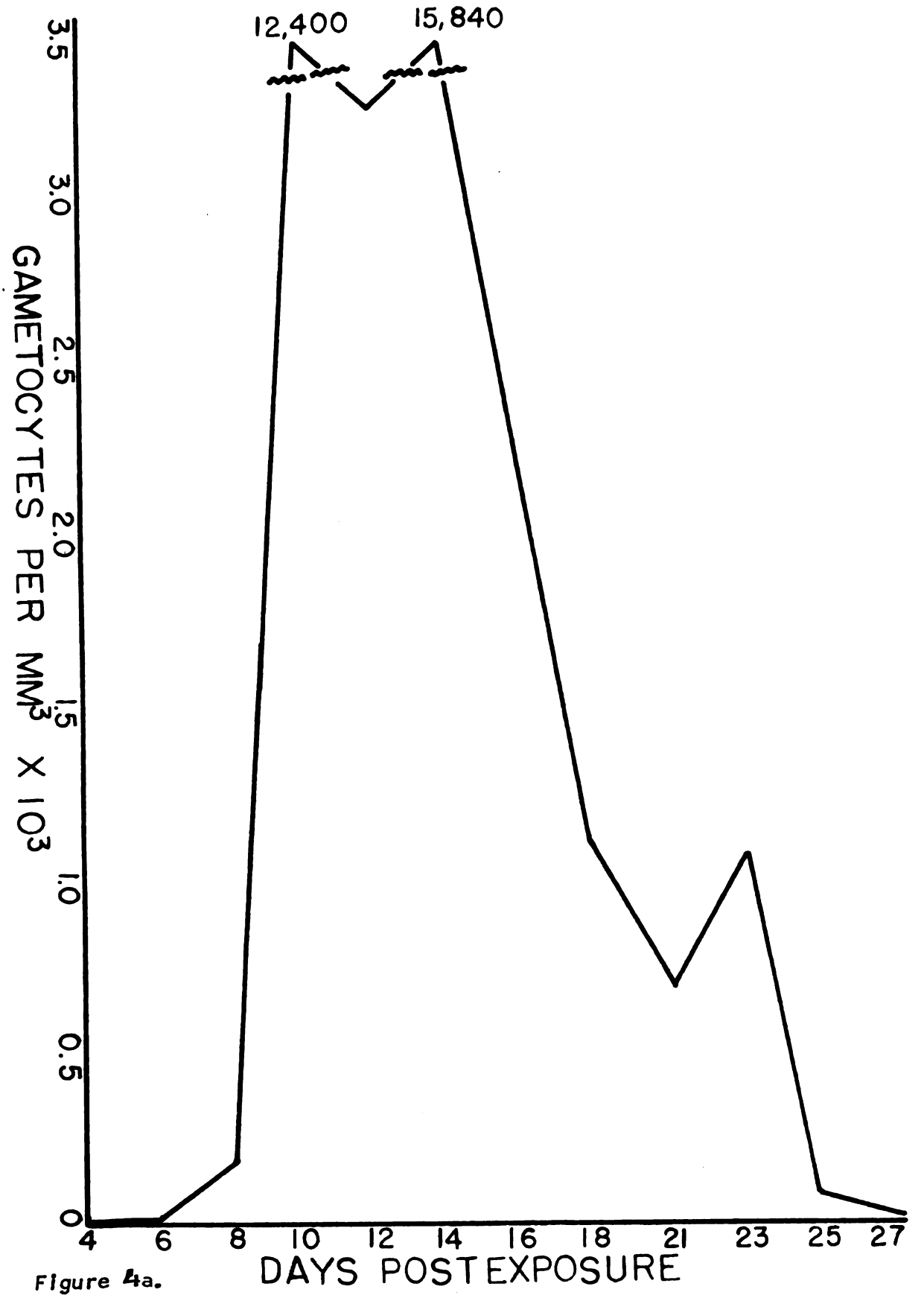
12,400
15,840
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
GAMETOCYTES PER MM3 X 10³
4
6
8
10
12
14
16
18
21
23
25
27
DAYS POST EXPOSURE

Figure 4a.

Figure 4b. Summary graph of Table II showing the mean gametocyte densities per mm^3 blood of the birds kept out of direct sunlight.

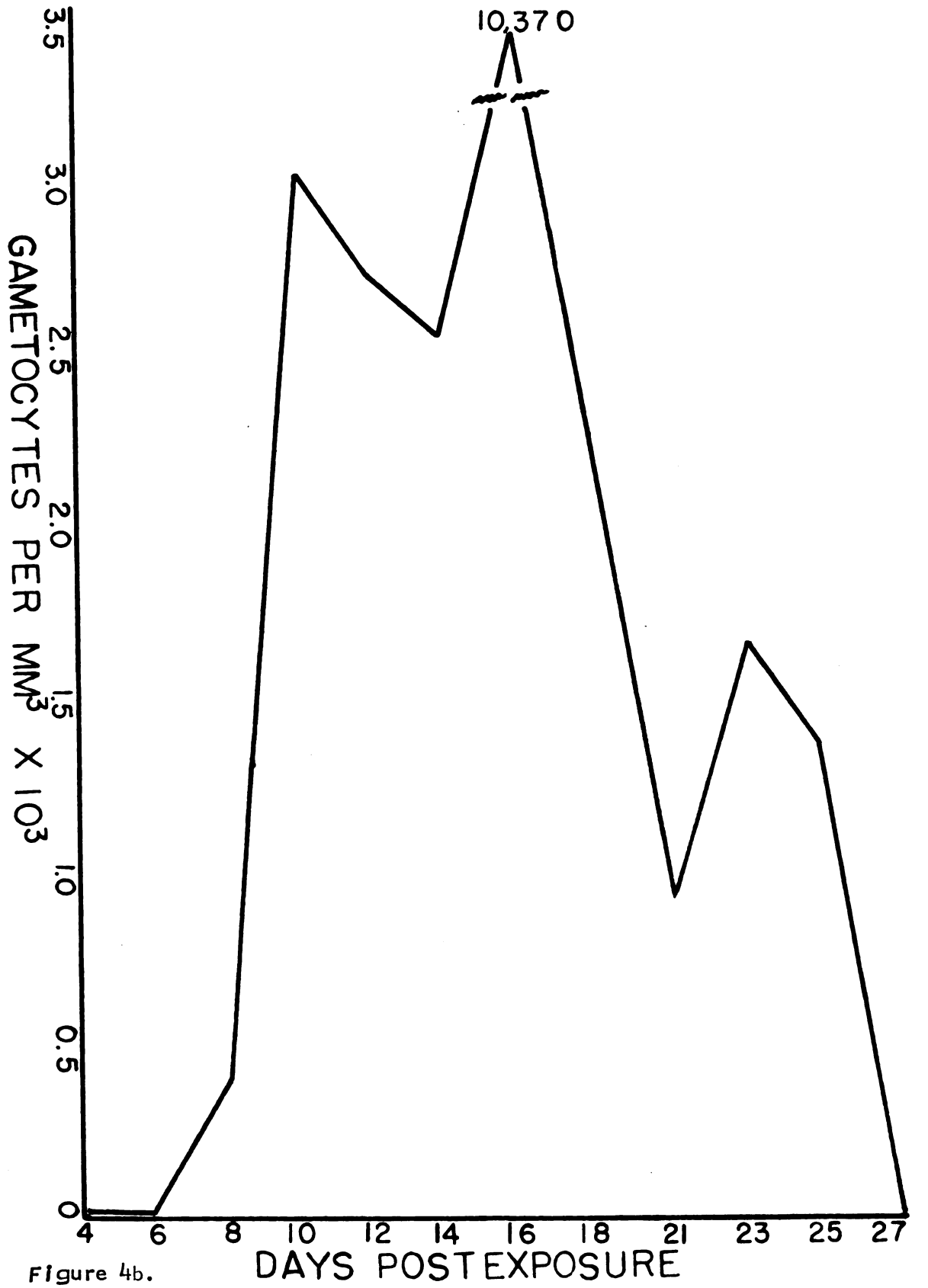
10,370
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
GAMETOCYTES PER MM3 X 10³
4
6
8
10
12
14
16
18
21
23
25
27
DAYS POST EXPOSURE

Figure 4b.

Table I. Number of gametocytes in infected controls kept under conditions identical to those of the experimental birds. Two birds show three gametocyte density peaks occurring on the tenth, fourteenth, and twenty-third day post-exposure. The third bird lacks only the first peak but is otherwise identical to the other birds.

	Days Post-Exposure									
Bird No.	8	10	12	14	16	18	21	23	25	27
2653	520*	22320	4650	13370	5987	3856	1664	1130	0	0
2655	105	14880	3330	12160	1820	1319	460	2170	620	0
2656	0	0	2000	21990	8350	1060	0	0	0	0
Average	208	12400	3326	15840	5386	2078	708	1100	206	0

Table II. Individual gametocyte counts of birds kept in a dark pen but allowed to experience normal day-night lighting. Comparing peak densities with the control birds one sees that the three peaks correspond nicely, with only a slight variation in the second peak.

2698	410*	3240	3640	1960	10450	2100	1505	3300	2830	0
2699	420	2780	1780	3100	10290	2400	318	0	0	0
Average	415	3010	2710	2530	10370	2250	912	1650	1415	0

*gametocytes per mm^3 blood

Figure 5. Average gametocyte density of four birds in Table III showing an obvious lack of the second and third peaks of gametocyte density.

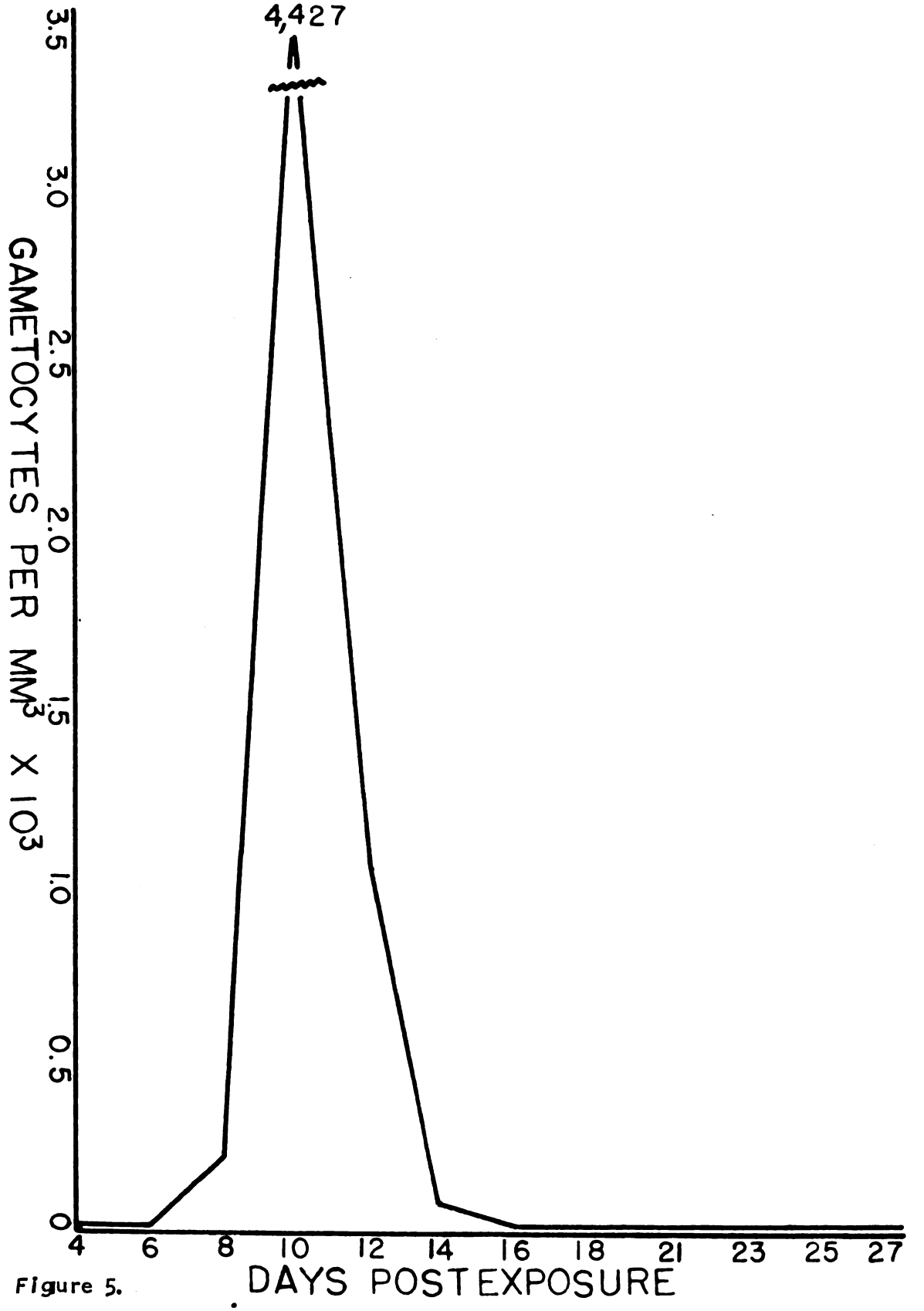
4,427
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
GAMETOCYTES PER MM3 X 10³
4
6
8
10
12
14
16
18
21
23
25
27
DAYS POST EXPOSURE

Figure 5.

Table III. Gametocyte densities of the four birds who demonstrated an apparent beneficial effect from being immunized with immune gamma globulin prior to infection. The single peak occurs at the same time as the first peak in the control group.

	Days Post-Exposure									
Bird No.	8	10	12	14	16	18	21	23	25	27
2681	820*	14580	1000	0	0	0	0	0	0	0
2682	0	1080	1600	0	0	0	0	0	0	0
2687	123	1880	1810	510	0	0	0	0	0	0
2688	0	170	0	0	0	0	0	0	0	0
Average	236	4427	1103	127	0	0	0	0	0	0

Table IV. Four birds from the group immunized prior to infection which showed no apparent effect from the immune gamma globulin. The peak gametocyte densities occur on the same days as do the other groups of birds.

2680	550*	21320	18600	10140	11900	5800	1188	1660	1070	381
2685	256	9100	3500	3030	6880	2220	0	810	0	0
2686	320	10440	2940	49200	18350	13200	1068	1080	1020	0
2689	0	12720	8880	15114	24800	13450	0	830	430	0
Average	281	13395	8480	19371	15482	8667	564	1095	630	95

*gametocytes per mm^3 blood

Figure 6. Summary graph of Table V showing the gametocyte densities per mm^3 blood of the birds immunized at the beginning of patency.

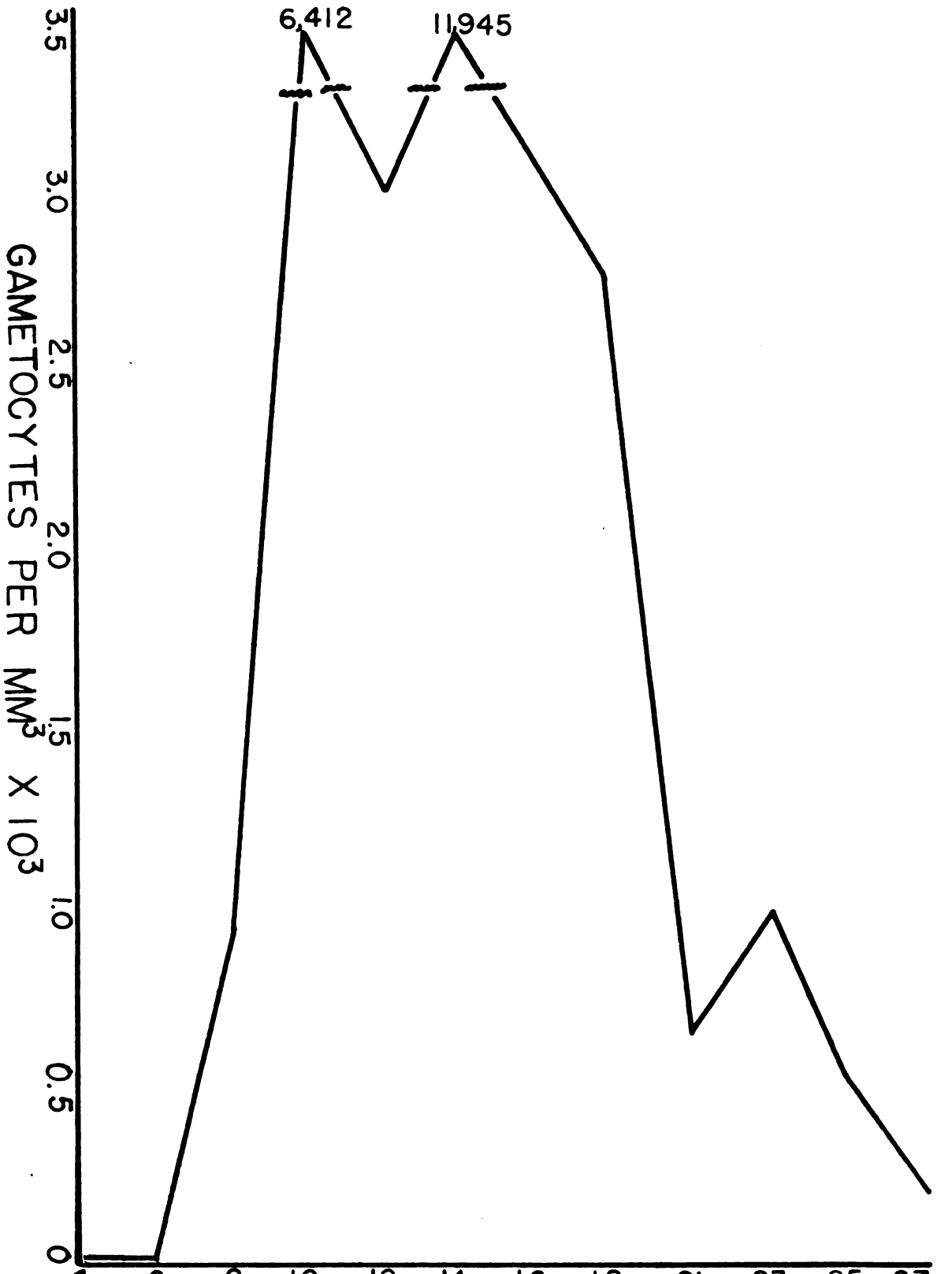
6,412
11,945
GAMETOCYTES PER MM3 X 10³
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4
6
8
10
12
14
16
18
21
23
25
27
DAYS POST EXPOSURE

Figure 6.

Table V. Gametocyte densities in the birds immunized with immune gamma globulin on the first day immature gametocytes appeared in the blood. It should be noted from the record of each bird and from the average of the entire group that the peak gametocyte numbers occurred on the tenth, fourteenth, and twenty-third day post-exposure. These times correspond closely with those of the control group.

	Days Post-Exposure									
Bird No.	8	10	12	14	16	18	21	23	25	27
2660	620*	6550	4653	15110	13650	6250	0	2030	690	0
2661	0	9900	1260	4680	3960	1620	0	1070	515	0
2662	700	1300	4530	5900	2640	1485	298	0	0	0
2665	0	0	425	3610	8950	778	0	700	0	0
2666	3780	17800	7640	17250	4880	3900	3280	2680	10900	1500
2668	204	3040	1300	24020	4080	3000	662	510	625	0
2669	1000	5800	1570	13050	1880	1910	60	0	0	0
Average	900	6412	3054	11945	5720	2706	614	998	533	214

*gametocytes per mm^3 blood

Very few birds lost more than 1 x 10^6 cells per mm^3 at any time during the course of the parasitemia but all birds demonstrated some degree of anemia, though variable, during the crises. Figures 7a and 7b demonstrate the lack of uniformity in the degree of anemia exhibited; however, they show that there tends to be some fluctuation in erythrocyte numbers in accordance with the parasite density when it remains low. Ducks with high gametocyte densities showed an anemia which began with the first appearance of gametocytes and lasted until recovery from the primary parasitemia. Those with low gametocyte densities showed low red cell counts which corresponded to the peaks of gametocyte density and recoveries which corresponded to the crises. By the last gametocyte crisis all birds showed a blood count within the range of the uninfected controls

Differential counts of the various forms of gametocytes showed a definite change in forms that corresponded to the changes in peaks and crises of gametocyte density. The per cent of each type of cell is indicated in Figure 8. It can be seen on comparison of the form graph and the parasite density graphs that immediately prior to each peak of parasite density there is a greater per cent of immature forms. This is very evident during the first two peaks but becomes somewhat obscured by the third peak. It will also be noted that the highest per cent of round gametocytes occurred at the first peak and decreased continuously as the infection progressed. The elongate forms first appeared in very low percentages twelve days post exposure, at the time of the first crisis. From this time on their levels became higher until by August 10th only elongate forms were seen.

The four birds in Group No. 1 which showed low parasite densities

Figure 7a. This figure depicts the concurrent but reciprocal fluctuation of gametocyte and erythrocyte numbers observed in some of the infected birds with low parasite densities.

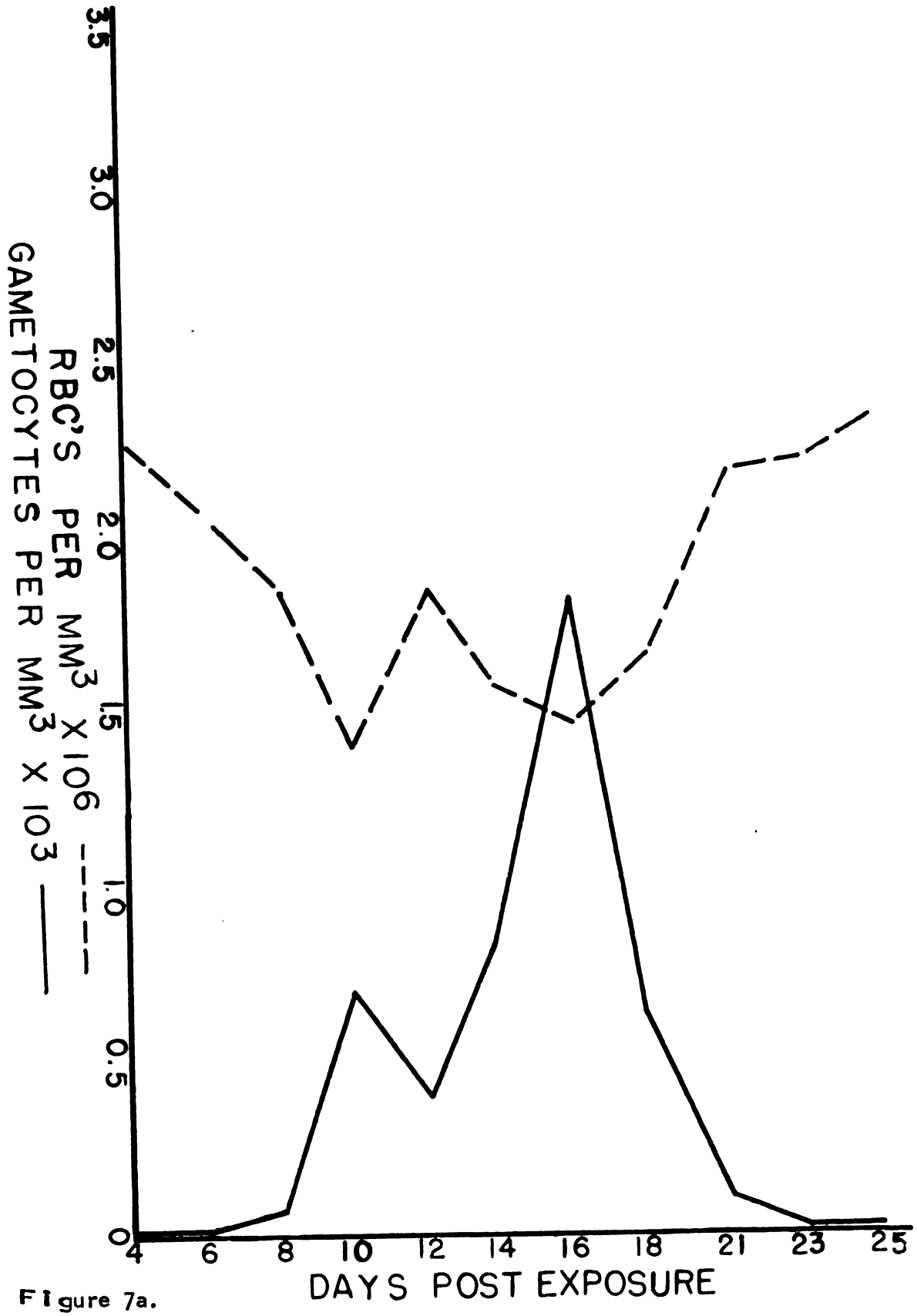
RBC'S PER MM3 X 106 ------
GAMETOCYTES PER MM3 X 103 ——
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
4
6
8
10
12
14
16
18
21
23
25
DAYS POST EXPOSURE

Figure 7a.

Figure 7b. This curve shows the commonly noted reciprocal gametocyte-erythrocyte densities at the time of the first gametocyte peak with a subsequent recovery of erythrocyte numbers in the presence of the second and higher gametocyte peak.

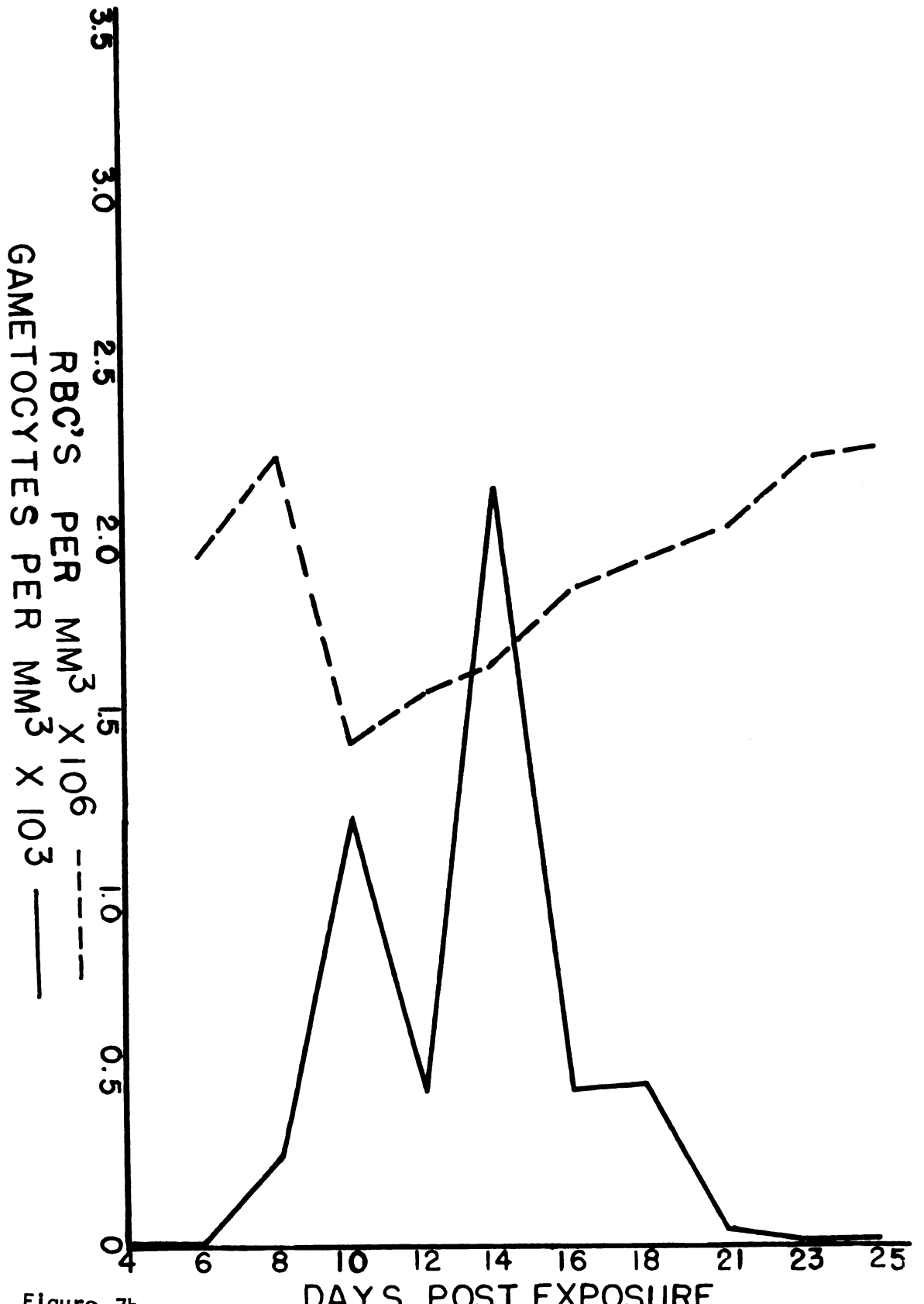
RBC'S PER MM3 X 106 ----
GAMETOCYTES PER MM3 X 103 ——
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
4
6
8
10
12
14
16
18
21
23
25
DAYS POST EXPOSURE

Figure 7b.

Figure 3. Average numbers of the three forms of gametocytes seen during the course of Leucocytozoon simondi infection. The three crosses at the top of the graph indicate the peaks of gametocyte density. The first peak is composed almost entirely of round forms while the second two peaks are composed predominantly of the elongate forms.

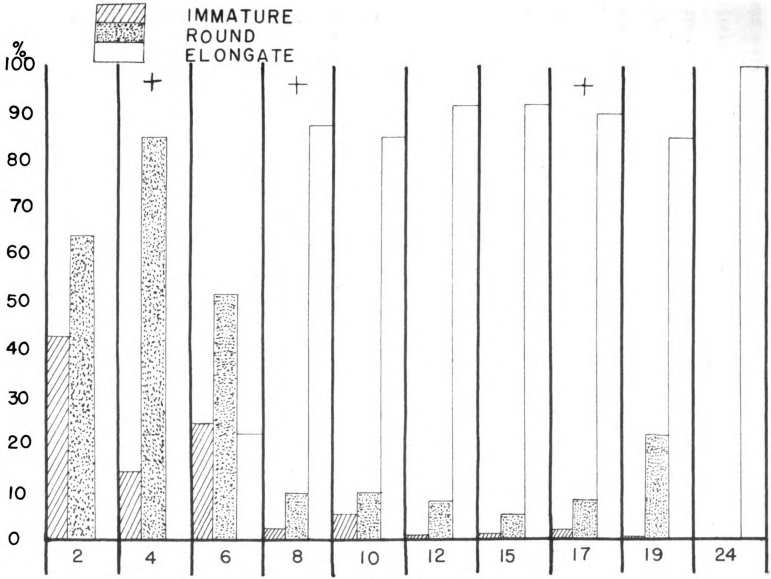
IMMATURE
ROUND
ELONGATE
%
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
15
17
19
24
Days Post-Exposure

Figure 8.

and a single peak, never showed elongate forms even though their parasites were evident beyond the time when all other birds showed these forms. One of the other four birds from this group, who showed an otherwise normal cycle, demonstrated a delayed appearance of elongate forms. It also showed a relatively low gametocyte density.

Close examination of the elongate forms at their high per cent levels showed a number of forms which appeared to be similar to the small immature or undifferentiated forms. Their percentages were not determined due to the uncertainty as to what they were and their distorted shape. Also noted was a number of round or polygonal parasites in elongate host cells (Figures 9a, 9b and 9c). On August 8th, twenty-two days after exposure, a change was noted in the appearance of the host cell nucleus of the elongate and some of the round forms (Figure 10). There appeared to be an erosion or breaking apart of the cell nucleus. This same phenomenon occurred in the first set of birds but at the time it went unrecorded because it was believed to be an artifact of staining or fixation.

A positive anti-globulin test was obtained two times during the primary infection of the two adult birds. Data as to duration of positive anti-globulin reaction and titer throughout the infection were not obtained due to a contamination noted in the rabbit anti-gamma globulin serum after the first two tests.

Figure 9a.

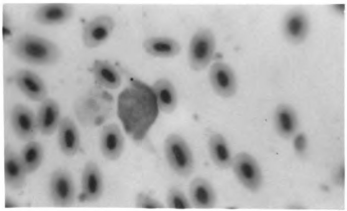

Figure 9b.

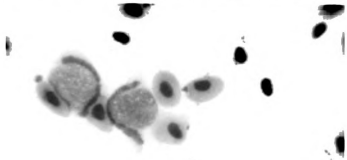

Figure 9c.

Figures 9a, 9b and 9c. Round and polygonal gametocytes of Leucocytozoon simondi in "elongate" host cells. Note the extensions of the host cell. (Giemsa).

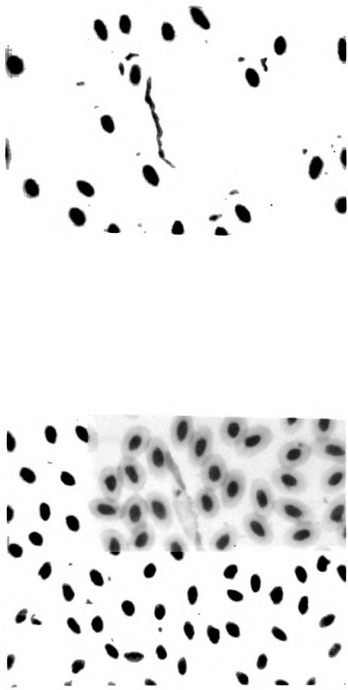

Figure 10. Photomicrograph of two gametocytes of Leucocytozoon simondi in host blood cells showing signs of erosion (Giemsa).

DISCUSSION AND CONCLUSIONS

A careful review of the work done to date on Leucocytozoon simondi reveals that there is a great deal yet to be clarified before a thorough understanding of the organism, in all of its aspects, will be possible. A discussion of the results reported above may serve to clarify some of the vague aspects of the biology of L. simondi.

In comparing the data obtained from the first set of experimental birds exposed on June 16th with those exposed on July 16th, it was noted that the duration of prepatency and patency were not the same in both groups. This phenomenon has been recorded several times. The first time was during the summer of 1963 while the author was working on a Leucocytozoon problem for Dr. J.H. Barrow at the University of Michigan Biological Station, Pellston, Michigan. The second occurrence was during the first set of experiments reported in this paper and again in 1964 by Dr. Barrow (personal communication). The birds observed at Douglas Lake and those reported by Barrow all showed extended prepatent periods lasting 10 or more days and terminated in the death of the birds. The birds exposed by the author in mid June, 1964 showed the same prepatent times but survived the initial parasitemia and subsequently recovered. Chernin (1952a) also reports that those birds exposed prior to the first fly feeding episodes and remaining in the endemic area for the duration of the transmission period showed a different parasite cycle in the host than did those birds exposed at a later date. He described this as being low level infection which did not appear until more than ten days post-exposure (Table VI). The extended prepatent period was probably somewhat obscured due to some amount of time in waiting for the appearance of the vectors. Only the prepatent period was

Table VI. Data collected by Chernin (1952a) from birds exposed to black flies at various times during the summer. Note the low mortality and extended prepatent period of those birds exposed early in the season and allowed to remain in the endemic area throughout the transmission period.

Experiment	No. of birds	Dates of exposure	% Infected	% Patent at 10 days	Fatalities
Permanent	11	6-16 to 8-24	100	0	0
I	11	6-29 to 7-7	82	11	0
II	12	7-11 to 7-19	100	100	83
III	15	7-19 to 7-27	93	64	14
IV	10	7-27 to 8-4	90	89	56
V	10	8-8 to 8-16	30	33	0
VI	10	8-16 to 8-24	0	0	0

involved in the work reported here since the birds were exposed for only twenty-four hours.

To what this extended prepatency could be attributed is open purely to speculation. If the gametocyte numbers are any indication of the number of sporozoites introduced, then the inoculum size could not account for this extended prepatent period. This is supported by comparing the gametocyte densities from the first set of birds with those of the second set.

An attractive explanation for this occurrence is that these infections are produced by the first infected flies of the season. Since the parasite has not been shown to be transovarially transmitted from fly to fly it is probably acquired anew each year by the newly emerged flies. The parasite's long residence in the duck, which is by this time at least a year long, may have attenuated the gametocytes to such an extent as to decrease their virulence and thus one sees an "abnormal" cycling in the first infections of the year. Once the parasite has been rapidly passed from vector to host and back to vector a number of times, which indeed does happen in endemic areas, the virulence of the organism is enhanced and one then sees a "normal" cycle in the host.

Another explanation may lie in the species vector involved in the transmission. During the early weeks of the summer a different species of Simulium may be transmitting the parasite while later in the summer a different species may be the vector, and indeed the vector at either time may not even be a Simulium but some other arthropod such as the recently suspected Culicoides. The possibility of different vectors finds support in Fallis' extensive work with these vectors which showed many species of Simulium to be capable of transmitting Leucocytozoon to

the Anserorida and Culicoides as being able to transmit Leucocytozoon to other avian species.

The time of exposure also affects the pattern of the disease in the vertebrate. In contrast to early summer exposures, midsummer exposures produce a gametocyte density fluctuation during the primary infection. Also necessary for detection of this fluctuation is a reliable counting method for determining gametocyte density in the peripheral blood. A method employed by malariologists, that of calculating the number of parasites per volume of blood, was used in this work in an attempt to get a truer and more reliable picture of the gametocyte density during the primary attack. As Figures 4a, 4b and 6 and Tables I, II and V show, a fluctuation in the density of gametocytes present in the circulating blood was detected. This phenomenon has not to this date been described for Leucocytozoon. It was suspected by Chernin and Sadun (1949) but was later repudiated by Chernin (1952c) on the basis of counting gametocytocytes per unit time. Fallis et al. (1951) presented a graph which is based on a similar counting method which shows three peaks which correspond nicely to those described in this paper (Figure 11). However, the degree of rise and fall in numbers is greatly reduced in this graph. This is probably due to the number of erythrocytes present in any given volume of blood varying during the sampling period while the speed at which the observer could count parasites remained essentially the same, except at extremely low and high parasite densities.

In the experiments reported here a standard exposure time of twenty-four hours was set and data analyses were based on these exposures. All of the figures showing the course of infection in the Pekin presented here show that the first sign of infection appears on the seventh day

Figure 11. A graph reproduced from Fallis et al. (1951) showing three distinct peaks in gametocyte numbers. These peaks correspond closely to those reported by the author but are based on one minute counts from blood smears.

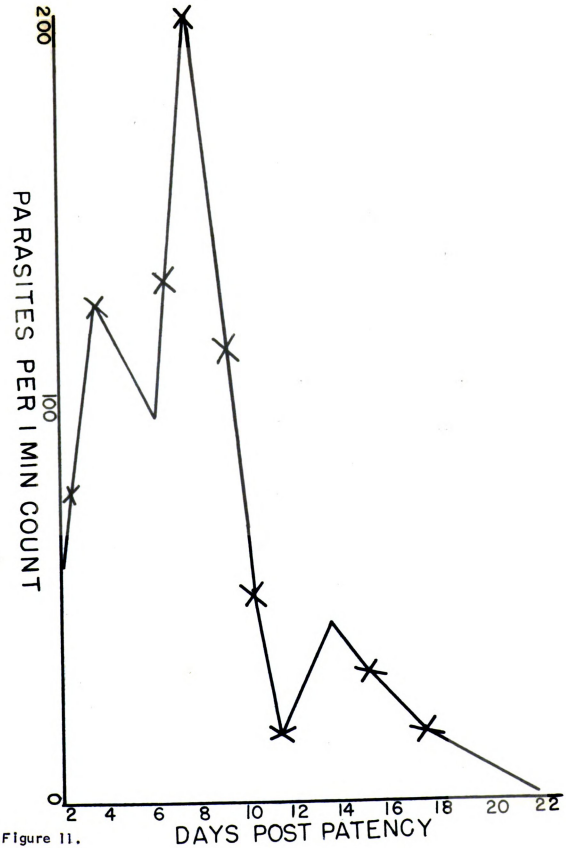
200
100
0
PARASITES PER I MIN COUNT
2
4
6
8
10
12
14
16
18
20
22
DAYS POST PATENCY

Figure 11.

post-exposure. Chernin's results show clearly that all those ducks exposed for a single twenty-four hour period became patent at the same time; whereas ducks exposed for extended periods show infections in which the beginning of patency varied considerably, although he did not interpret his results this way.

A factor in favor of single exposures is seen in the consistent appearance of the pleomorphic gametocyte forms. Although these will be discussed in greater detail later in this paper it should be noted here that when ducks are exposed for a single day the elongate form of the gametocyte appears on the same day in all birds exposed at the same time. On the other hand, birds exposed for more than one day show a parasite picture with variable appearance of the elongate forms (Figure 8 and Table VII). If these forms are a part of the maturation cycle of one species of parasite then it seems logical to expect them all to appear at the same time if their precursors were all introduced into the host at the same time.

Another point which also advances the idea that single exposures give truer pictures of the vertebrate cycle is the very synchronous gametocyte fluctuations which occur during the course of the primary infection.

The fluctuation in gametocyte levels does not appear to be the result of sequestering of cellular elements with their subsequent release. This is borne out by the observation that in some cases the number of erythrocytes was at a low point while the gametocyte density was at a high point. The possibility of preferential or specific sequestering of the parasite from the blood should not be overlooked. In Plasmodium falciparum infections such a sequestering of blood forms

Table VII. Chernin's table (1952c) showing the consistent appearance of the elongate gametocytes on the fifth day of patency in birds exposed for 24 hours as compared to the variable time of appearance of these forms in birds exposed for extended periods.

	immature peak	appearance of 1st elongates	elongate peak	total peak
one day exposures	4	5	8	8
	4	5	9	9
	4	5	7	6
	3	5	6	3
average	3.8	5.0	7.5	6.5
8 day exposure	5	5	9	9
	5	3	6	7
	2	1	5	4
	5	5	9	8
	4	5	9	4
	5	12	16	5
	6	5	10	6
	4	4	6	6
	6	6	12	8
	3	4	9	6
	3	6	8	3
average	4.4	5.1	9.0	6.0

does indeed take place. However, the forms removed are the maturing schizonts, not the gametocytes. Since no circulating schizonts have been shown to occur in L. simondi infections this would be a unique situation if the gametocytes were removed.

Another, and more likely explanation, is that gametocytes are being released en masse from the tissue megaloschizonts at specific times during the course of the infection. The prepatent asexual development proposed by Fallis et al. (1956) consists of two asexual cycles prior to gametocyte release. Since gametocyte patency usually occurs on the 6th to 8th day post-exposure an asexual cycle of 3 to 4 days in length is likely. If this same cycling continued beyond that first appearance of gametocytes it would be expected to produce a high percentage of immature forms and peaks in gametocyte density about 3 to 4 days apart; and indeed that is exactly the picture seen in Figures 4a, 4b, 6 and 8 and Tables I and II. Cowan (1955) stated that he believed the megaloschizonts to be the sole contributors to the gametocyte population seen in the blood. If this is true then the hepatic schizonts in the Kupffer cells of the liver must be the precursors of the first megaloschizonts. The question now arises: Do the megaloschizonts produce only gametocytes or both gametocytes and more megaloschizonts? If they do not produce more megaloschizonts, then the hepatic schizonts must survive and divide during the remainder of the infection or are capable of reproducing themselves. If one of these suggested possibilities were not true then it would be difficult to explain the presence of relapse gametocytes more than a year after the initial exposure to sporozoites. Another possibility is that the megaloschizonts produce in addition to gametocytes, a merozoite similar to the sporozoite which then invades the liver and begins the cycle over from the

beginning. Figure 12 shows the hypothetical cycling possibilities which could explain the fluctuation in gametocyte densities and the presence of parasites after recovery from the initial acute infection. If this type of cycling was the case it would be reminiscent of Coccidial infections. Eimeria tenella, a parasite of chickens goes through a development which is very similar to 'b' in Figure 12. Following infection by sporozoites there occurs a first generation schizont. This produces a second generation schizont which is the precursor to gametocytes and a third generation schizont. The third generation schizont produces gametocytes and very rarely another schizont generation. This development would be analogous to the hepatic schizont of Leucocytozoon producing megaloschizonts which are the source of gametocytes and other megaloschizonts.

A third explanation for the occurrence of the peaks in gametocyte levels has its basis in the work of Cowan (1955, 1957) and Fallis et al. (1951). These workers demonstrated megaloschizonts in various tissues during the course of the primary infection. As the infection progressed, more and more of the tissues became negative for megaloschizonts indicating that they had either ruptured and spilled their merozoites into the host or that the host had destroyed them with some immunological mechanism, as described by Cowan (1957). If they were disappearing due to their rupture upon becoming mature, then it is possible that megaloschizonts in different tissues mature at different rates and therefore release gametocytes at different times during the infection.

The fourth possibility is the presence of two or even three species or strains of Leucocytozoon with the same host range and specificity. If such a situation did occur it is conceivable that the vectors would

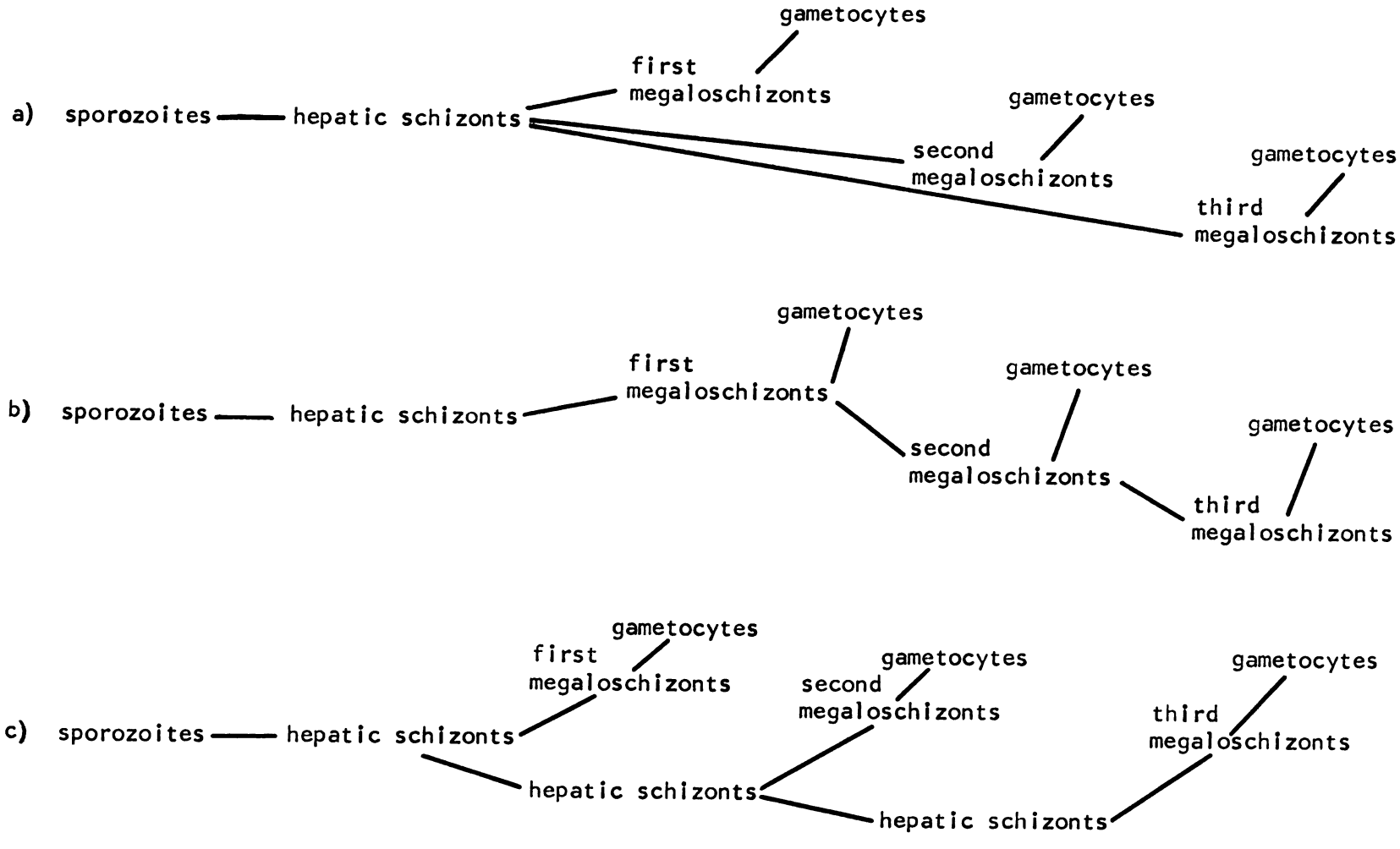

Figure 12. Author's hypothetical routes of Leucocytozoon simondi development in the duck based on existing known facts about the life cycle.

be infected with two or three of these species or strains and that transmission of all of them occurred simultaneously. The report by Chernin (1952c) that four ducklings went through an entire cycle without showing a difference in gametocyte morphology is interpreted by some to be an indication of a single species of parasite.

If the different species or strains had different maturation times this would account for the gametocyte peaks occurring at different times but in coincidence with each other. The presence of different species or strains in the same host may result in a type of competition and/or inhibition of one by the other. If this were the case then the cycles of these different parasites would be out of phase with one another and their maximum gametocyte densities would be at different times.

The reduction in number of circulating gametocytes by the thirtieth day of infection would most likely be the result of the host's immune mechanisms keeping these forms in check. This will be discussed further in the section on immunity. On the other hand, it may be due to a mechanism similar to that seen in various Eimeria species in which a characteristic number of schizogonies occur after which the infection is terminated.

The investigators in this area have not made use of the techniques of malarial research even though the two organisms are suspected to be closely related. Standardization of inoculum or exposure time in Leucocytozoon research is practically unheard of though it is common practice among malariologists. A second error in results of experiments with this organism lies in the determination of the parasite density in the bird host. This has previously been done by counting the number of parasites in a standard thin blood smear observed during some given

period of time. A second commonly used method is to count the number of parasites per microscopic field or per some given number of erythrocytes. It becomes obvious that these methods must be improved before an accurate picture of the course of the infection in the vertebrate host can be elucidated.

It seems improbable that sporozoites introduced into a single host over a period of a week will all be in the same stage of development at the same time. If they all develop at approximately the same rate some will be ahead of others in their maturation, thus a single exposure of short duration (12 hours) would give the most accurate picture of the parasite's course in the vertebrate.

Unless exposure and counting techniques are standardized it will be difficult to control other phases of research with this organism. The results of the passive immunity experiments reported above would not have been observed unless these conditions were met.

Since reference to immunity to Leucocytozoon is rarely found in the literature, it was necessary to turn to Plasmodium research for ideas on how to attack the problem of humoral protection to Leucocytozoon. The majority of the literature on plasmodial immunity deals with cellular immunity since it has been believed for many years that humoral antibody to malaria was nonexistent or at most a nonprotective factor. The work reported by Cohen and McGregor (1963) on passive transmission of humoral immunity to Plasmodium falciparum in Gambian infants provided an excellent basis on which to develop methods for studying this same phenomenon in Leucocytozoon infections. The methods used to passively immunize ducks to Leucocytozoon were similar to those used in the above reported work. The results, however, manifested themselves

in quite a different way from those results obtained by the two authors.

As mentioned under results, when immune globulin was given to infected ducks at the first sign of parasites in the blood, there was no effect noted. The infections proceeded in a manner very similar to the infections of the untreated control birds (Figure 6). However, when immune globulin was administered immediately prior to exposure to the vectors there occurred a lower number of gametocytes in the peripheral circulation and a loss of the second two peaks of gametocyte density in half of the birds as compared to the gametocyte population noted in the controls. There also appeared to be no alteration in the degree of anemia in the birds who showed reduced parasitemias. This point is contrary to the reports of Cohen and McGregor (1963) since they demonstrated the alleviation of all symptoms of the disease with the exception of pyrexia.

Since the nature of perpetuation of the parasite in the tissues has yet to be elucidated it is impossible to know just where the humoral protection might act.

One explanation for the lower gametocyte number is that it was not due to transferred immunity at all, but was a normal variation in the vertebrate cycle of the parasite. It seems unlikely, however, that of all of the birds used this should occur in half of the treated birds in one group and in none of the other birds examined during the experiment. Since other authors used different methods of determining gametocyte density it is impossible to compare other work in this case.

If the lowered gametocyte density was due to a passively transferred immunity, the results can be explained. The results obtained in malarial immunity studies have led investigators to speculate that the humoral protection acts against the schizonts or against the merozoites when

they are in an extracellular state. The latter suggestion seems most likely since the schizonts remain inside their erythrocyte until rupture of the parasite occurs. Based on these postulates one can see that when the earliest forms appear in the circulation in Leucocytozoon infections they are the result of previous schizogony and have already entered their host cell and are therefore protected against attack by humoral antibody. It also becomes apparent that the schizonts in Leucocytozoon infections are even less susceptible to humoral antibody attack than are the erythrocytic schizonts of malaria, since they are entirely within the internal organs. Even when acquired immunity has eliminated essentially all of the blood forms of malaria, the tissue schizonts remain unharmed, as is the case with recoveries from Leucocytozoon. The survival of the tissue schizonts could be due to their being nonantigenic -unharmed by the antibody produced against them- or because they are not in contact with the antibody produced. Clark (1964) showed that the internal organs of the magpie, infected with a species of Leucocytozoon, were positive for gametocytes even when the peripheral blood was negative. This indicates that production of gametocytes or at least survival of them continues throughout the life of the host but the host is capable of suppressing their entrance into the peripheral circulation. From the results of the birds immunized at the first sign of peripheral stages, it can be seen that the immune globulin does not work against the gametocytes. Based on these results it must be assumed that the protection noted in the pre-exposure treated birds must be the result of antibody action on the prepatent stages. This immune action might well be against the tissue stages, but in untreated birds humoral immunity does not manifest itself until patency has first been noted

(Cowan, 1957). If Cowan is correct in interpreting the results he obtained from histological studies of the tissue stages at the onset of patency, then it must be assumed that humoral protection occurred before this time in the immunized birds. If the prepatent asexual cycle proposed by Fallis is correct then it is possible to speculate on just where the immunity does work.

Starting with the first proposed asexual generation, the immune action may have been toward the entering sporozoites or against the hepatic schizonts themselves. If it was against the sporozoites then there would be fewer or attenuated hepatic schizonts. If the action was against the schizont or against its newly released merozoites, then the megaloschizont generation would be interrupted. This again might manifest itself in lower numbers or in attenuated gametocytes. The last place that immune globulin might act to alter the course of the infection in the vertebrate host is at the megaloschizont, as Cowan suggested, or on its progeny, the merozoites.

Referring back to the postulated tissue recycling, a number of possibilities or combinations of these may represent the susceptible stage in the cycle. If as mentioned above the reduction in numbers of gametocytes and the lack of the last two peaks in density were a normal variation in the cycle of the disease, then further discussion is beyond the scope of this paper. However, if they are the result of the immunization then some discussion as to how these phenomena came about is in order.

If the first explanation for the peaks in gametocyte density, that of preferential sequestering, were true, then the immune globulin might well enhance the ability of the host to sequester and retain the gametocytes within the tissues. Since gametocytes have not been shown to

play a role in the pathology of this disease, then this mechanism would not play a protective role in the defense against the parasite. Looking at the entire waterfowl population, however, it is obvious that if there were a low pool of gametocytes for the vectors to draw from then transmission should be reduced to where sporozoite numbers would be below a critical level. Such a mechanism is substantiated by the low mortality rate in wild populations.

The second proposed explanation for the gametocyte density peaks is an internal cycling which releases merozoites at regular intervals. Any action on the tissue stages or the merozoites which they release would have an effect on the later gametocyte population. If the action were against the merozoites which were destined to produce megaloschizonts then the lower gametocyte numbers would be a direct result of fewer megaloschizonts, and indirectly the result of immune action on their precursors.

The possibility of there being different rates of megaloschizont development in different tissues might go hand in hand with the possibility of different tissues being more susceptible to infiltration by humoral globulin. If this were true, the gametocyte fluctuation could be explained in this way: Certain of the tissues are invaded by the merozoites which rapidly develop into megaloschizonts while they are relatively well shielded from immune attack. Other megaloschizonts, developing in other tissues, would be maturing more slowly and would be more susceptible to the action of immune globulin. This situation would yield the early gametocyte peak from the rapidly developing and protected megaloschizonts. The gametocyte picture which would result from this series of happenings would be a single peak occurring at its expected

time and the inhibition of development of the gametocytes for the succeeding peaks.

If the fourth possibility for gametocyte fluctuation were true, that is that more than one species or strain of Leucocytozoon might be involved the possibility arises that one of these strains might be more susceptible to the host's defense mechanisms than the others. If the above proposal is correct and the peaks in gametocyte density are due to the different rates of development of the various strains or species of the parasite, then when the host's antibody destroys one or more of these the antibody titer against it gradually diminishes until by the time the serum for immunization is collected the titer is insufficient to protect the immunized birds. Based on this possibility, the course of the gametocytes in the immunized bird would be as follows: The second and third peaks which do not appear in the immunized birds are the peaks produced in the unimmunized birds by the parasites which are not completely eliminated by the host's defense mechanisms. The result is high antibody titer resulting from premunition, the constant stimulation by the residual parasites. The first peak would represent those parasites which were completely eliminated by the unimmunized host. Since the titer was declining at the time of serum collection there was incomplete protection conferred on the immunized birds, or if the titer was so low as to produce no protection, the low peak may be a result of cross immunity between the species or strains which survived and that one which was eliminated.

Birds which survive the primary parasitemia produce a latent infection. This is most likely the result of an acquired immunity obtained during the course of the initial encounter with the parasite. As

Clark (1964) reported the number of gametocytes in the tissues far exceeds the number noted in the circulation during periods of latency. This would indicate that there is little or no immune inhibition of the production of gametocytes but that they are being retained within the tissues where they were produced. Whether or not they can survive indefinitely once they have been produced has not been demonstrated. Indications tend to exclude their continued existence beyond some short time. Fallis et al. (1951) showed that gametocytes transplanted into clean hosts survived in the circulation only 6 to 7 days. Also, if all gametocytes produced during the period of latency were to be retained in a viable state throughout the year then why does the relapse the following spring show only 1/1000 the gametocyte density as did the primary infection?

It is possible that gametocytes are produced and destroyed at a constant rate during the latent period and are released into the circulation by some mechanism during the relapse noted in the spring.

All of the defense mechanisms of the host may not always be to its advantage. Many authors have suggested that in protozoan infections, and in particular, malarial infections, there is the possiblity of an autoimmune mechanism acting in the host.

The observation that anemia occurs during L. simondi infections is frequently reported; however, as mentioned above, accurate erythrocyte counts were not made routinely and the infections studied were multiple infections by the same organism resulting from long exposure, thus tending to blend and/or obscure any accurate picture of the anemia.

Comparing the erythrocyte counts with the gametocyte densities in Figure 4a and Tables I and II it becomes obvious that the high and low peaks of gametocytes correspond inversely or directly. Comparing the

anemia with the course of infection in the vertebrate, one can see that those figures referred to for comparison of anemia with parasitemia show a relatively low parasite level.

The above described coincident rise and fall in erythrocyte and gametocyte levels could be interpreted to be the result of some common cause. Since it is quite evident that the circulating gametocytes could not cause such a drop in erythrocyte numbers the next most obvious cause would be the tissue stages. There is good evidence, as mentioned above, that the megaloschizonts are responsible in some way for the fluctuations in gametocyte levels, but these stages present no obvious means of causing anemia. As will be recalled from the section on results, a positive anti-globulin test was noted on several occasions during the infection. This will be discussed in greater detail in the next section on immunology but it might be noted here that this could readily cause an erythrocyte destruction as the result of an autoimmune response. The tissue stages may be responsible for the autoimmune response and loss of erythrocytes. This mechanism could account for the seeming coincident fluctuations of gametocyte density and erythrocyte numbers.

Zuckerman (1964) put forth a number of possible explanations for such autoimmune responses, some of which would do well to explain the erythrocyte loss in L. simondi infections. Since it is still unknown as to which type or types of cells are involved as infected host cells in this infection, it is more difficult to postulate just why the erythrocytes become Coomb's positive. One or more of the following proposals may be the answer: 1) The possibility of a common antigen or a similar antigen would explain the positive test. That is to say, the parasite possesses some antigen, which is different enough from those of the host

to elicit an antibody response but is still similar enough to the host's erythrocyte antigens to cause the antibody to react with the erythrocytes. A similar situation is seen in the treatment of rabies victims with rabbit tissue from the CNS. In some cases the patient's immune response to the rabbit tissue also acts on his nervous system in a way which destroys a portion of the myelin sheath. 2) Another explanation may lie in the possible alteration of the host's tissue or possibly the exposure of certain antigens which normally are not in contact with the immune mechanism of the host. In this case the host reacts with his own tissue to produce an antibody against a part of its normal antigenic make-up. The alteration or exposure of their antigens would be the result of the parasite's penetration of the host's cells. 3) The third explanation is the adsorption of parasite antigen on the surface of the host's erythrocytes. These antigens may be metabolic products of the parasite released into the extracellular spaces of the host or may result from the breakdown of whole parasites. In either case, the antibody response toward these antigens on the surface of the host's cells would appear to be a response against the cells themselves. 4) The altered ability of the host to recognize self from non-self would also produce such an autoimmune response. In this case the host produces antibodies against its own tissue. This possibility may have grounds in the supposition of a number of authors who state that the invaded host cells are cells of the lymphoid series. Since these cells are known to play a role in antibody production it is conceivable that they may be altered to such an extent by the invasion that they react against the host's own antigens.

Obviously, from the four possibilities for autoimmune responses mentioned above, there is no one solid definition of an autoimmune response.

As it is used here it will be considered the apparent destruction of the host's own tissue as a result of its own immune responses.

This autoimmune response usually manifests itself in the form of anemia or in the destruction of some vital tissues of the host. As mentioned above, a positive anti-globulin test was demonstrated during the course of an L. simondi infection in an adult Pekin. Since this test was negative for uninfected controls it was assumed that the reaction was the result of the infection. Zuckerman (1964), however, has recently shown that the reticulocytes produced during anemia also exhibit a positive Coomb's test. This makes the above results somewhat doubtful. At least a new test must be employed to clarify the proposed autoimmune response.

If an autoimmune mechanism is not involved and some other means of erythrocyte destruction is involved, then the graphs showing gametocyte and erythrocyte fluctuations (Figure 7) might be interpreted to show that the host can compensate for erythrocyte loss very rapidly providing that the parasite burden is not too great. When the gametocyte levels are high, indicating a heavier parasite burden, there appears an initial drop in erythrocyte numbers which remains at a low level until recovery. The host apparently can not compensate for the loss.

The suggestion by Cook (1954) that erythrocyte destruction is due to their penetration by merozoites seems completely without grounds. If this destruction was due to parasite invasion, what can account for the loss of one million or more erythrocytes when only 5 to 20 thousand parasites are present in an equal volume of blood. One argument might be that the destruction is occurring in the internal organs. What then would be the mechanism of destruction since it is well established that

no schizogony occurs in the erythrocytes and no evidence has been put forth that the gametocytes change host cells during their residence therein. Another argument might be that the stem cells of the erythrocytic series are being invaded. If this were true one could not explain the rapid recovery from anemia three consecutive times. A single recovery would be plausible due to the storage of erythrocytes in the bone marrow, but it is difficult to conceive of three rapid recoveries when the stem cells are being destroyed and are unable to replenish the storage cells.

Immunity or autoimmunity may also be involved with other cell lines in the host, such as the leucocytes.

Although no experiments were designed to elucidate just why there was more than one host cell type and parasite shape, there appeared in the results of this work a phenomenon which may be of some value in clarifying this question. In reference to Figure 8, it can be seen that on the sixth day after the onset of patency and at the first gametocyte crisis, there appeared the so-called elongate gametocytes. From that time on they increased in per cent until by the onset of latency they constituted 100% of the gametocyte population. Figures 2, 9 and 10 show elongate forms which were observed during the course of the blood slide examinations. There appeared to be more than just the elongate gametocyte involved in these spindle shaped host cells. Some are immature gametocytes, others are the commonly observed round or polygonal shaped gametocyte, and the elongate gametocyte. The reports that no transitional stages exist between the round and elongate forms seen in the blood are not borne out by these photographs. There are obviously round gametocytes enclosed in an elongate host cell.

Just what causes the pleomorphic forms has not been shown. Some authors suggest the difference to be in the type of host cell invaded, but as Cook (1954) pointed out, if this were true the two forms should appear at the same time. Chernin's suggestion (1952c) that it may be the result of the host's immune response is not supported by the results obtained in the immunization experiments reported above. No difference was noted between the immunized and control birds in the appearance or number of elongate forms. One exception to this is noted in the four birds which showed only one low gametocyte density peak. At no time did elongate forms of the gametocyte become evident during the course of the blood examinations of these four. However, if this were the result of the immune globulin acting on the host cell-parasite complex, the same type of reaction would be expected to occur in the birds immunized when the gametocytes first appeared since the antibody titer would be higher in these birds at the time the gametocytes were released. In fact, this lack of elongate forms did not occur in any other birds in these experiments.

Another suggestion by Chernin (1952c) is the influence of fundamental host differences. This could account for the various forms and could also be responsible for the single peak in gametocytes discussed above. His third suggestion, that of parasite strain differences also presents a likely possibility for explaining the variation in gametocyte forms.

Briggs' report (1960), that Muscovy ducks showed less than 5% elongate forms and suffered much less severely from Leucocytozoon infections, compared with the apparent lack of elongate forms in the immunized ducks mentioned above does indeed suggest that some type of immunity may be the cause of this phenomenon. Since good evidence has been presented which rules out humoral influence on this occurrence the possibility arises

that cellular sensitization may produce the difference. This could be explained if during the early part of the infection macrophages or other phagocytic cells are becoming sensitized to parasite antigen while phagocytizing it, and later when they come in contact or are invaded by a merozoite this elongation response manifests itself. This mechanism is purely speculative but it does have possibilities.

SUMMARY

Two-week-old Pekin ducklings were exposed for twenty-four hours to infected black flies so as not to produce concurrent infections of Leucocytozoon simondi originating at different times which might obscure the true course of a single infection in the duck host.

Red blood cell counts and gametocyte densities per mm^3 of blood were made during each experiment.

Two sets of birds were exposed, the first on June 15th and the second on July 15, 1964. There were obvious differences in the gametocyte picture and duration of the prepatent and patent periods in the two sets of birds. Prepatency in birds exposed early in the season lasted 14 days followed by a steady increase in gametocyte numbers and a subsequent crisis and recovery. The birds exposed in midsummer demonstrated a seven day prepatency and a gametocyte density which was observed to have a periodicity with three peaks, one on the 4th, 8th to 10th and 14th to 16th days of patency. The significance of the peaks is treated in the paper.

Another observation which suggested the possibility of a periodicity existing in the vertebrate cycle of this parasite was the appearance of the so-called "elongate" gametocytes. These first appeared in all but one bird on the sixth day of patency. Elongate host cells containing round gametocytes were observed and interpreted to be intermediate forms possibly resulting from a previous sensitization of the host's cells.

An attempt was made to passively immunize ducks against Leucocytozoon using gamma globulin from recently recovered ducks. None of the birds immunized on the first day of patency showed a deviation of parasitemia when compared to control birds. Half of the birds immunized immediately

prior to infection demonstrated a loss of the second two peaks of gametocytes and a drastically lower number of gametocytes when compared to the controls.

It is suggested that anemia which accompanies infections of Leucocytozoon may be due to an autoimmune response of the host. It has been demonstrated in several instances that fluctuations in gametocyte density and anemia occur simultaneously.

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