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Heterologous Immunity in Avian Hosts Infected with *Plasmodium relictum* and *Plasmodium lophurae*

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Abstract. The course of *Plasmodium relictum* infections in 21-day-old White Leghorn cockerels was followed. The levels of parasitemia were extremely low (0.01 to 0.18 per cent) and the duration shor (one to four days). Subsequently, when *P. lophurae* was injected into chickens previously infected with *P. relictum*, a partial immunity was observed. Although the specific duration of this protection was not determined, it did persist for at least 30 days.

The previous inoculation of *P. lophurae* into pigeons afforded some protection against subsequent *P. relictum* infections, suggesting the presence of a reciprocal immunity.

Previous explanations of cross-immunity have included virulence of the parasite, duration of infection, and common antigens. Neither *P. relictum* nor *P. lophurae* were virulent, nor did they persist for long in pigeons or chickens. Accordingly, the heterologous immunity observed between *P. relictum* and *P. lophurae* is considered to be a function of common antigens.

Research in this Laboratory concerning immune reactions of bursaless and normal chickens, indicated the possibility of cross-immunity between *Plasmodium relictum* and *P. lophurae*. The development of immunity to malaria is in itself a controversial subject, and cross-immunity is even more so. In the past, several workers have reported cross-immunity between strains (TALIAFERRO and CANNON 1936; MANWELL and GOLDSTEIN 1938) and between different species of plasmodia (GINGRICH 1932; MANWELL 1938, 1940), while others have failed to show it (HARTMAN 1927; MANWELL 1929; KIKUTH 1931).

An observation made by FARMER (1965) was that if *P. relictum* infected blood was withdrawn from the donor pigeon before the crisis (pre-crisis) and then injected into chickens, the parasites persisted in the blood for several days and caused a low grade infection, but if the infected blood was drawn following the crisis (postcrisis), *P. relictum* failed to develop in chickens. The term 'crisis' has been variously defined (TALIAFERRO and CANNON 1936) and has been used to imply a time during an

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infection when protection of a non-specific nature becomes specific. In this study the term 'crisis' will be used to denote that time at which the development of parasitemia first reached its highest level.

In the light of suggestions that there is a direct relationship between the virulence of parasites (REDMOND 1939), duration of the infection (SINTON 1939), and the development of immunity, it was assumed that postcrisis *P. relictum* produced less immunity. The obviously rapid removal of post crisis *P. relictum* parasites from the hosts' circulation may have adversely limited their ability to activate the chickens' immune mechanism. For this reason, precrisis *P. relictum* have been routinely used in most of the experiments.

The present study was undertaken principally to investigate whether or not cross-immunity existed between *P. relictum* and *P. lophurae*. The experiments were designed to determine (a) the presence of cross-immunity between the two species, (b) the presence of a reciprocal immunity, and (c) the persistence of protection afforded by *P. relictum*.

MATERIALS AND METHODS

White Leghorn cockerels ranging in age from two to three weeks were used in this study. Therefore, throughout the remainder of this report, the term 'chicken' will refer to cockerels. However, the chickens used in each experiment were always of the same age, housed together, and handled as similarly as possible.

The parasites used were (1) a strain of *P. relictum* isolated from a mourning dove in 1960 and maintained by periodic blood passage in pigeons (FARMER and MOORE 1962), and (2) an agametocytic strain of *P. lophurae* maintained in chickens by periodic blood passage.

Parasite doses were calculated according to the weight of the chickens, parasitemia of donor, and total blood cell counts. The final dosage was estimated to deliver 100 million parasitized erythrocytes per kilogram body weight. Birds to be injected were inoculated by way of the alar vein.

The experimental designs were as follows:

Experiment 1. Eight two-week old chickens were inoculated with a volume of infected blood calculated to contain 10^8 pre-crisis *P. relictum* parasitized erythrocytes per kilogram body weight. Eight other chickens of the same age and approximate weight were randomly selected and were designated as controls, since they did not receive *P. relictum* infected blood.

Three days later another eight chickens were inoculated with a volume of infected blood calculated to contain 10^8 post-crisis *P. relictum* parasitized erythrocytes per kilogram body weight.

Ten days after the inoculation with pre-crisis *P. relictum* and, therefore, seven days after the injection of post-crisis *P. relictum*, five animals from the pre-crisis group, three from the post-crisis group, and five controls were all injected with a volume of infected blood calculated to deliver 10^8 *P. lophurae* parasitized erythrocytes per kilogram body weight.

Experiment 2. Twenty-four three-week old chickens were inoculated with a volume of blood calculated to deliver 10^8 *P. relictum* parasitized erythrocytes per kilogram body weight. Three days later, six of these chickens were injected with *P. lophurae* (10^8 parasitized erythrocytes per kilogram body weight).

Ten days following the original inoculation, another group of six chickens was injected with *P. lophurae*. The same dosage level was used for four more chickens inoculated 17 days after the original inoculation. The infective dose used was the same throughout the experiment.

Since six chickens of Experiment 2 had died during the inoculation procedures, eight more chickens were injected with pre-crisis *P. relictum*, using a volume of infected blood to deliver 10^8 parasitized erythrocytes. Three weeks later these birds were injected with pre-crisis *P. lophurae*. It should be pointed out that the animals used during this Experiment were not from the same hatch as those used in Experiment 1, but were the same age.

Experiment 3. Twenty-four 21-day-old chickens were inoculated with pre-crisis *P. relictum*. The infective dose was calculated to deliver 10^8 parasitized erythrocytes per kilogram body weight.

Six chickens were injected with pre-crisis *P. lophurae* seven days after being inoculated with *P. relictum*. A second group of six chickens was injected with pre-crisis *P. lophurae* 14 days after being injected with *P. relictum*. Another six chickens received injections of pre-crisis *P. lophurae* 21 days following injections with *P. relictum*. A final group of six birds was injected with pre-crisis *P. lophurae* 30 days after the initial *P. relictum* inoculation.

All *P. lophurae* infections were induced using a volume of infected blood measured to deliver 10^8 per kilogram body weight.

Experiment 4. Twenty-four 21-day-old chickens were inoculated with uninfected whole blood of a pigeon. Each bird received 0.25 ml of blood. This level of inoculum approximated that used to inoculate the birds used in Experiment 3. Then, following the same experimental scheme of Experiment 3, groups of six chickens were injected with pre-crisis *P. lophurae* seven, 14, 21 and 30 days after the initial injection of whole pigeon blood. *Plasmodium lophurae* infections were induced using a volume of blood calculated to deliver 10^8 parasitized erythrocytes per kilogram body weight. Capillaries of blood were collected daily for serum electrophoresis.

Experiment 5. Six pigeons (Modena strain) were inoculated with pre-crisis *P. lophurae* using 10^8 parasitized erythrocytes per kilogram body weight as an infective dosage. Eleven days later, these pigeons and four uninfected pigeons were infected with pre-crisis *P. relictum* calculated to deliver 10^8 parasitized erythrocytes per kilogram body weight.

During all the experiments, thin blood smears were made daily. These slides were fixed in absolute methanol for four minutes. After air drying, the slides were stained in Giemsa (1.0 ml stain: 20 ml distilled water) for a period of 20 minutes. The stained slides were examined using oil immersion. Parasites were enumerated according to the method of GINGRICH (1932), expressing parasitized erythrocytes per 10^4 erythrocytes counted. In those cases where parasite counts were low, 30 fields were examined to confirm a negative slide.

RESULTS

Experiment 1

Very low level parasite infections developed in the blood of eight two-week old chickens which had been infected with pre-crisis *P. relictum*. During the course of infections, the parasitemias reached a mean of 0.15 per cent parasitized cells on day five of the infection. The parasites were seen in the peripheral blood of seven out of eight birds until day six. In one bird, the parasites persisted until day seven. Another eight two-week old chickens were inoculated with post-crisis *P. relictum*. Parasites persisted in the blood for only one day and were no longer seen by day two of the infection (Tab. 1).

Ten days later, i.e., ten days following the inoculation of post-crisis *P. relictum* the recovered chickens of both groups and a control group of five previously uninfected chickens were inoculated with *P. lophurae*.

Table 1. The course of pre-crisis and post-crisis infections of *P. relictum* in two-week old white Leghorn chickens*

	Day of Infection							
	1	2	3	4	5	6	7	8
Pre-crisis								
Parasitemia (%)	0	0	0	0.08	0.15	0.09	0.01	0
Post-crisis								
Parasitemia (%)	0.075	0	0	0	0	0	0	0

Lower parasite levels and *P. lophurae* infections of shorter duration were observed in those animals previously inoculated with *P. relictum* than in those animals not previously inoculated (Fig. 1). Infections of *P. lophurae* in animals of all groups were compared. Infections in animals of the pre-crisis group persisted four days but developed higher parasitemias (18.0 per cent) than infections in postcrisis animals (11.8 per cent). Infections of both groups peaked on day two. The chickens of the postcrisis group developed the lowest mean parasite numbers, with the duration of infection being five days.

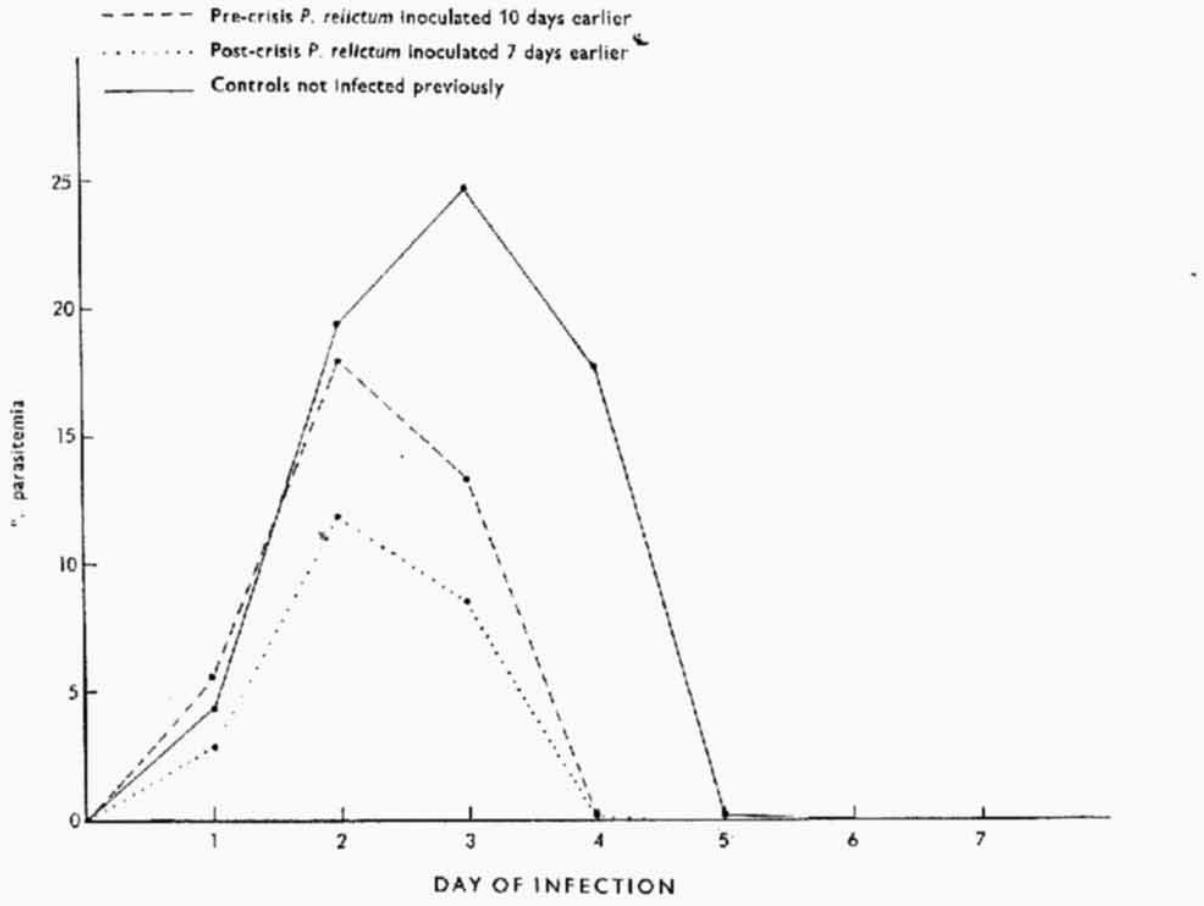

Fig. 1. The course of *P. lophurae* infections in 24-day old White Leghorn chickens. The inoculating dose contained 10^8 parasitized erythrocytes per kilogram body weight.

Finally, three chickens that had been inoculated with pre-crisis *P. relictum* infected blood and three not previously inoculated, were injected with *P. lophurae* infected blood. The time between the two inoculations was 19 days. The *P. lophurae* infection ran a rather mild course, reaching a peak mean of 7.9 per cent parasitized

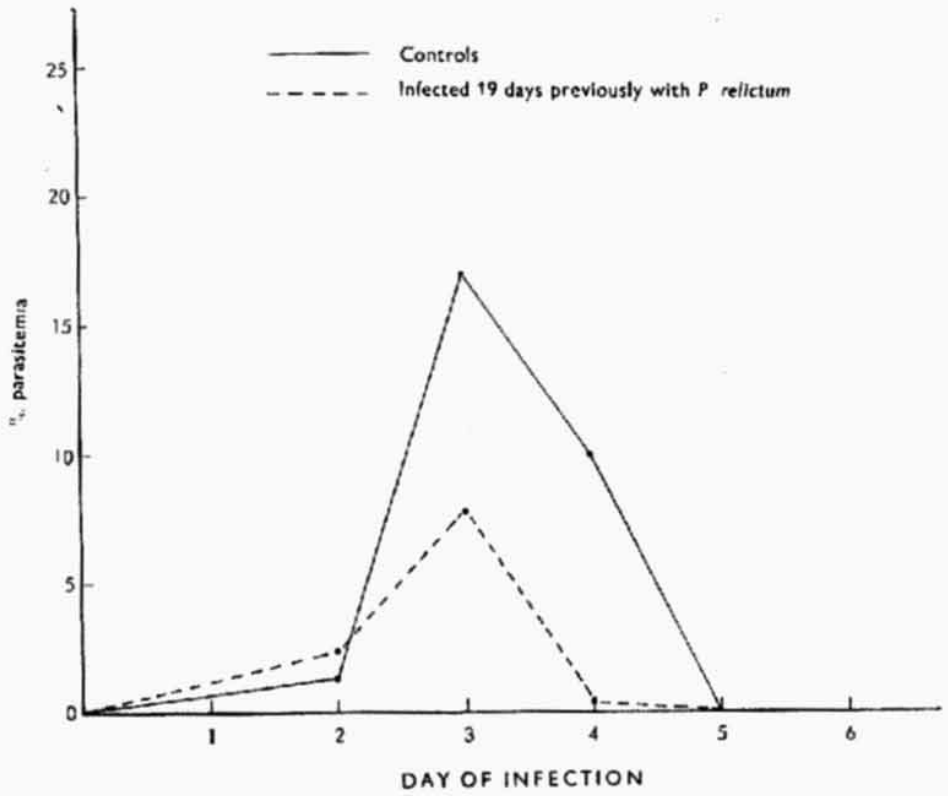

Fig. 2. The course of *P. lophurae* infections in 40-day old White Leghorn chickens. The inoculating dose contained 10^8 parasitized erythrocytes per kilogram body weight.

cells on day three of infection in the birds previously inoculated with *P. relictum* and persisting for six days. The parasitemias in the non-treated group also reached a peak on day three, but had a mean of 17.0 per cent parasitized cells. The infection was of five days duration (Fig. 2).

Experiment 2

Twenty-four three-week old chickens were inoculated with pre-crisis *P. relictum* parasitized blood. Three days later, six of these birds were infected with 10^8 *P. lophurae* parasitized cells per kilogram body weight, and the course of infection was followed. Low level *P. lophurae* infections developed in birds of this group, peaking at 7.30 per cent on day two. The infection persisted for five days (Fig. 3).

Six other chickens were chosen from the original group and inoculated with *P. lophurae* ten days following the initial infection. Low level *P. lophurae* infections developed, peaking on day three, with a mean of 4.2 per cent parasitized cells. The blood of all the birds except one was negative on day five of the infection. Parasites were no longer seen in the peripheral blood of that one bird by day six (Fig. 3).

Six more chickens from the original group were infected with *P. lophurae* 17 days after the original inoculation of *P. relictum*. Parasite levels were higher in this group, peaking on day three with a mean parasitemia of 22.10 per cent parasitized cells. The duration of infection in five of the six birds was seven days. The sixth animal developed an extremely unusual course of infection. The prepatent period was markedly delayed. Then, while parasitemias were decreasing in five birds

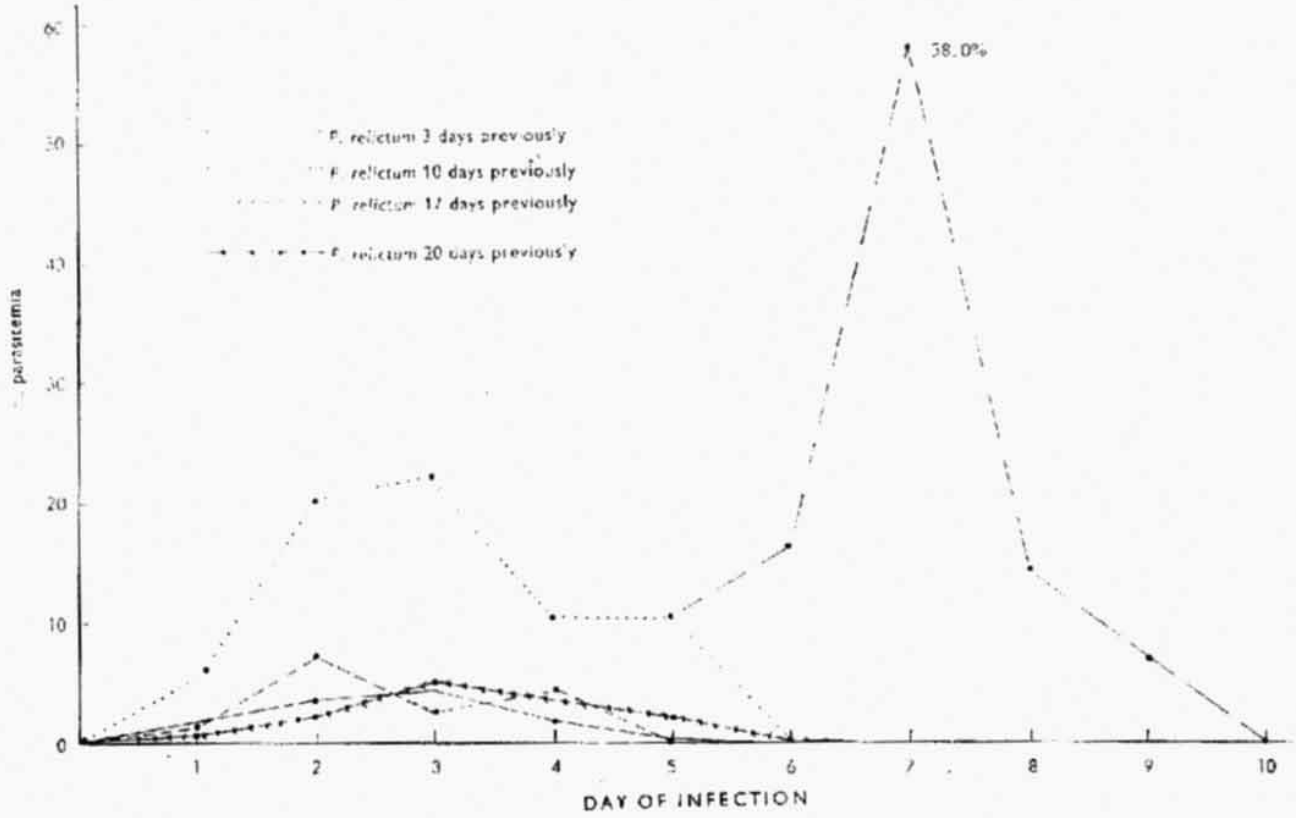

Fig. 3. The course of *P. lophurae* infections in 21-day old White Leghorn chickens which had been inoculated 3, 10, 17 and 20 days earlier with *P. relictum*. The inoculating dose of *P. relictum* and of *P. lophurae* were 10^8 parasitized erythrocytes per kilogram body weight.

after day three, parasite levels increased in this one bird from day five with 58.0 per cent parasitized cells being recorded on day seven. Parasites were no longer seen by day ten. The course of infection in this particular bird has been included in Fig. 3.

Since six birds of Experiment 2 had died, another eight chickens three weeks old were inoculated with pre-crisis *P. relictum* parasitized blood. Twenty days later these animals were inoculated with *P. lophurae*. The levels of parasitemia were quite low, peaking at day three with a mean of 5.0 per cent parasitized cells. The infection persisted for seven days (Fig. 3).

Experiments 3 and 4

Twenty-four three-week old cockerels were infected with pre-crisis *P. relictum*. From these 24 chickens, a group of eight were picked at random for observation of parasitemia and for obtaining blood for electrophoresis. The *P. relictum* infections

observed persisted, at very low levels, for four days, reaching a mean peak of parasitemia (0.18 per cent parasitized red blood cells) on day three (Tab. 2).

Six chickens, inoculated with *P. relictum* infected blood seven days previously, were infected with *P. lophurae*. Six chickens, inoculated with uninfected pigeon blood seven days previously, were also infected with *P. lophurae*. The courses of infection were observed and are compared in Fig. 4. The parasitemia peaked

Table 2. The course of *P. relictum* infection in eight three-week old white leghorn chickens*

	Day of Infection					
	1	2	3	4	5	6
Parasitemia (%)	0	0.075	0.180	0.075	0	0

on day three in both groups of animals and the blood of all birds was free of parasites by day seven; however, chickens inoculated with pigeon blood only, developed higher parasitemias throughout the course of the experiment and the infection persisted longer (eight days). In addition two birds of this group died during the infection. Apparently, *P. relictum* alone afforded protection against subsequent *P. lophurae* infections, whereas the pigeon blood did not.

Twelve chickens, six inoculated with *P. relictum* and six with blood, were all infected with *P. lophurae* infected blood 14 days after the initial inoculations. The

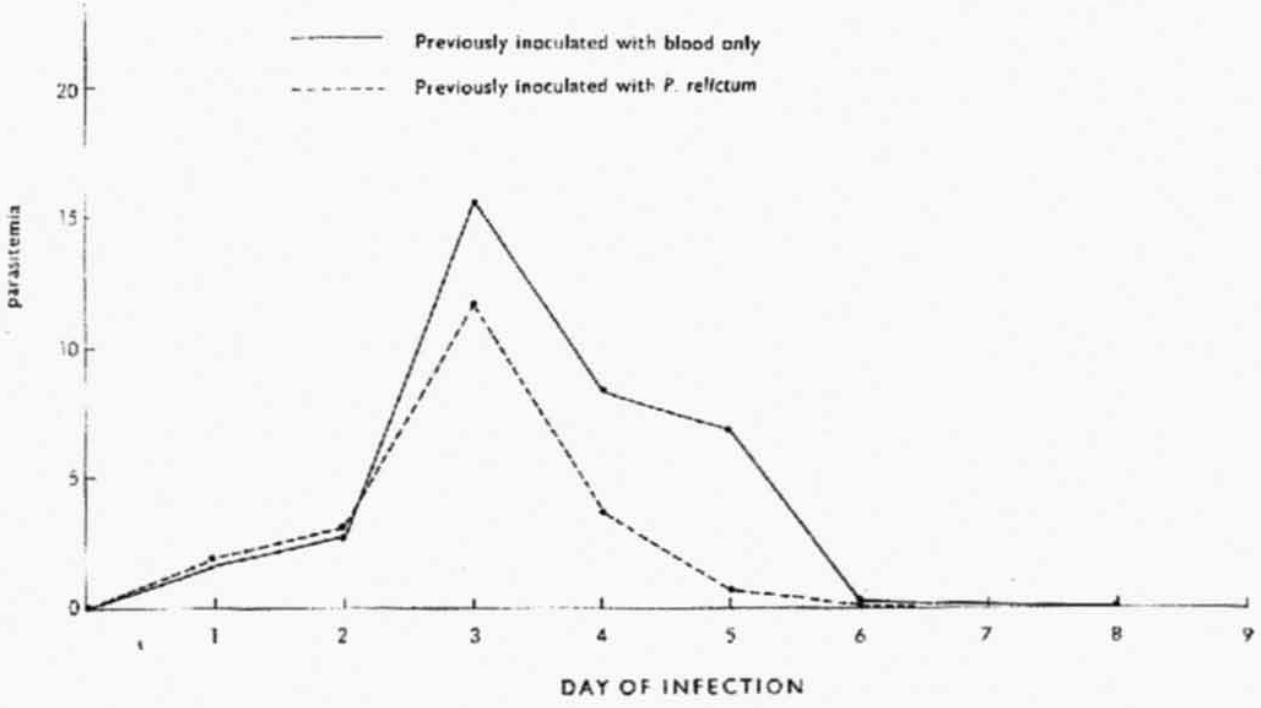

Fig. 4. The course of *P. lophurae* infections in four-week old White Leghorn chickens. The inoculating dose contained 10^8 parasitized erythrocytes per kilogram body weight.

courses of *P. lophurae* infection were followed in all birds (Fig. 5). The duration of infection (seven days) and day three peak of parasitemia (32.6 per cent in controls and 25.6 per cent in *P. relictum* recovered birds) coincided for both groups of birds. However, those animals previously infected with *P. relictum* experienced milder courses of infection. Even so, two birds of this group died as a result of malaria. Three of the other groups died from the effects of the infection.

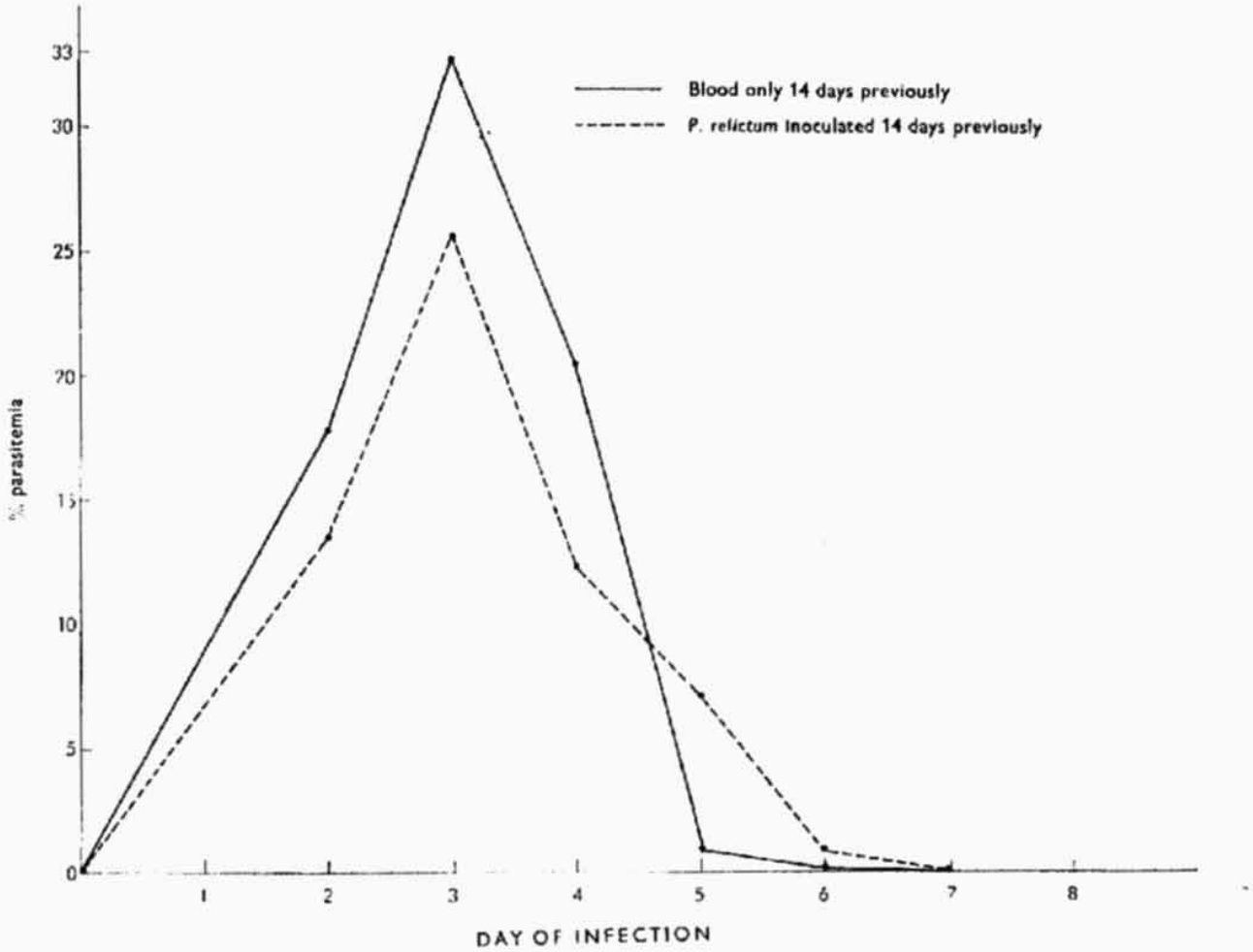

Fig. 5. The course of *P. lophurae* infections in five-week old White Leghorn chickens. The inoculation dose contained 10^8 parasitized erythrocytes per kilogram body weight.

Twelve chickens, six of which had been inoculated with *P. relictum* infected blood and six with uninfected blood, were injected with *P. lophurae* 21 days later. The course of infections was followed and recorded (Fig. 6). Parasitemias recorded from those chickens inoculated previously with whole blood only, peaked on day four with a mean parasite level of 31.04 per cent parasitized cells. Those previously inoculated with *P. relictum* developed much milder infections, peaking on day five with a mean parasite level of 19.60 per cent parasites. These data may not reflect a completely normal course of development since each of the groups of animals included an exceptional bird, one which developed a high terminal parasitemia. These are included in the data used for Fig. 6.

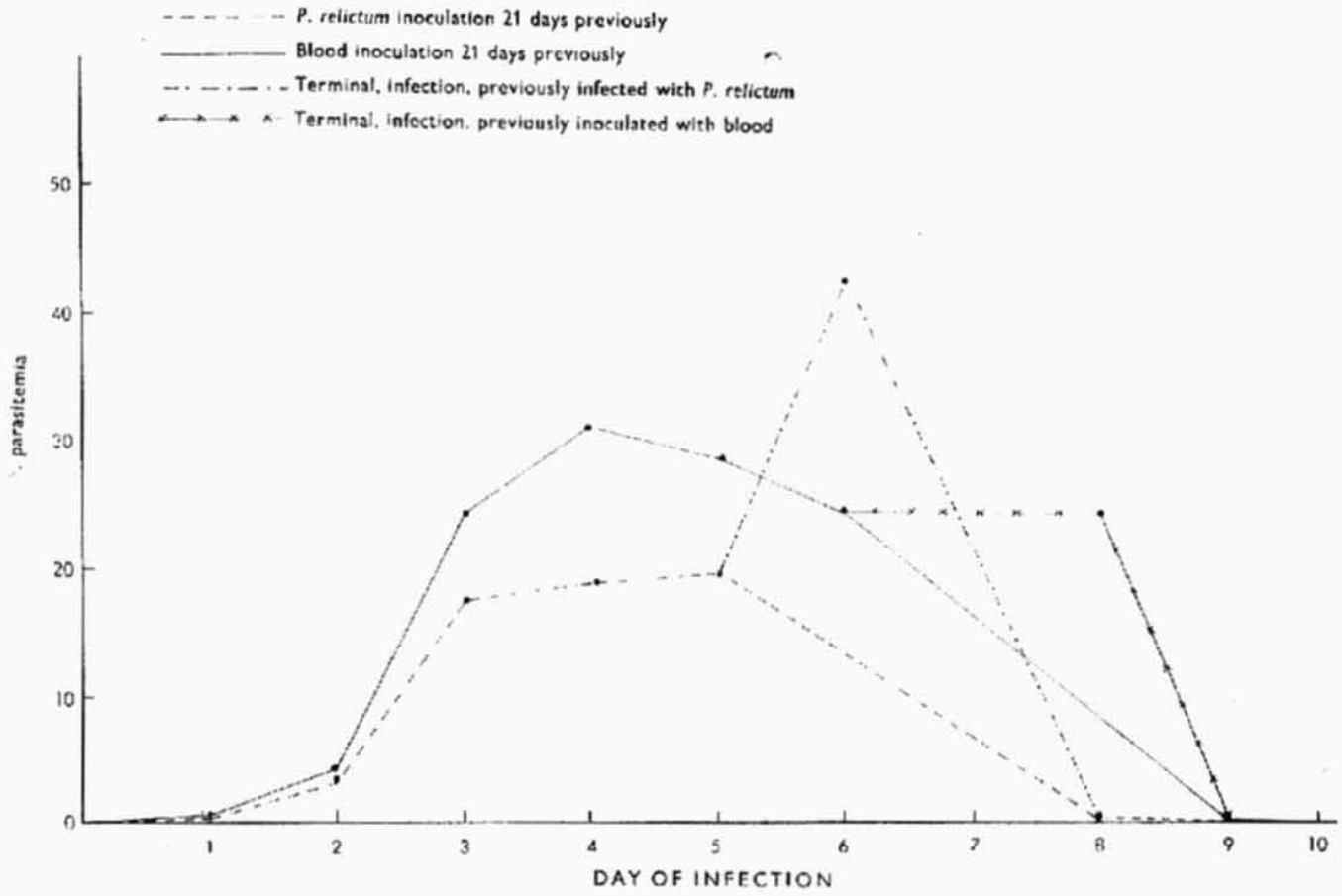

Fig. 6. The course of *P. lophurae* infections in six-week old White Leghorn chickens. The inoculum contained 10^8 parasitized erythrocytes per kilogram body weight.

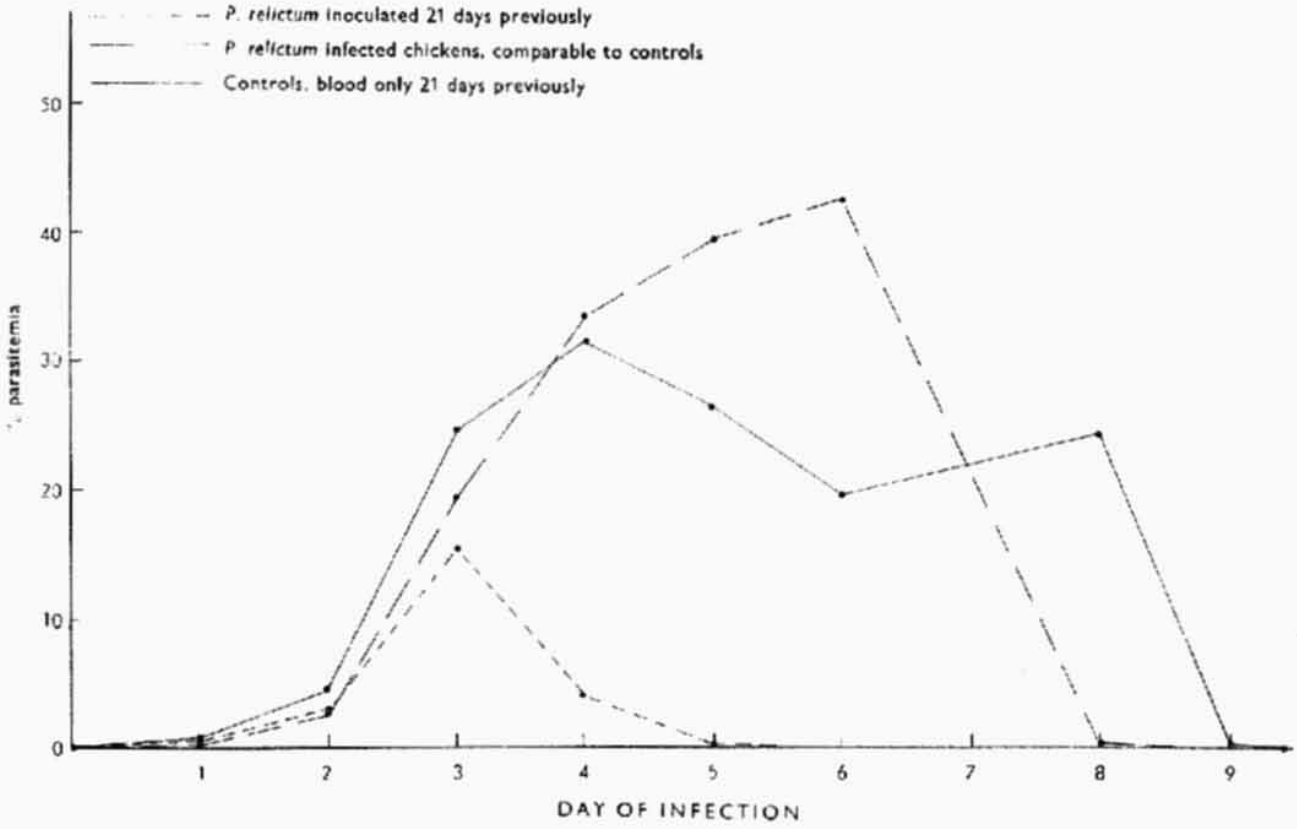

Fig. 7. Two varying courses of *P. lophurae* infections in chickens inoculated with *P. relictum* 21 days days previously. The inoculum contained 10^8 parasitized erythrocytes per kilogram body weight.

Another interesting aspect of this experiment was the observation associated with the course of infections and subsequent parasitemias. The group of animals previously inoculated with *P. relictum* was obviously divided into two groups. One group (i.e., half of the experimental group) exhibited very definite protection against *P. lophurae*. The parasites increased in numbers during the prepatent

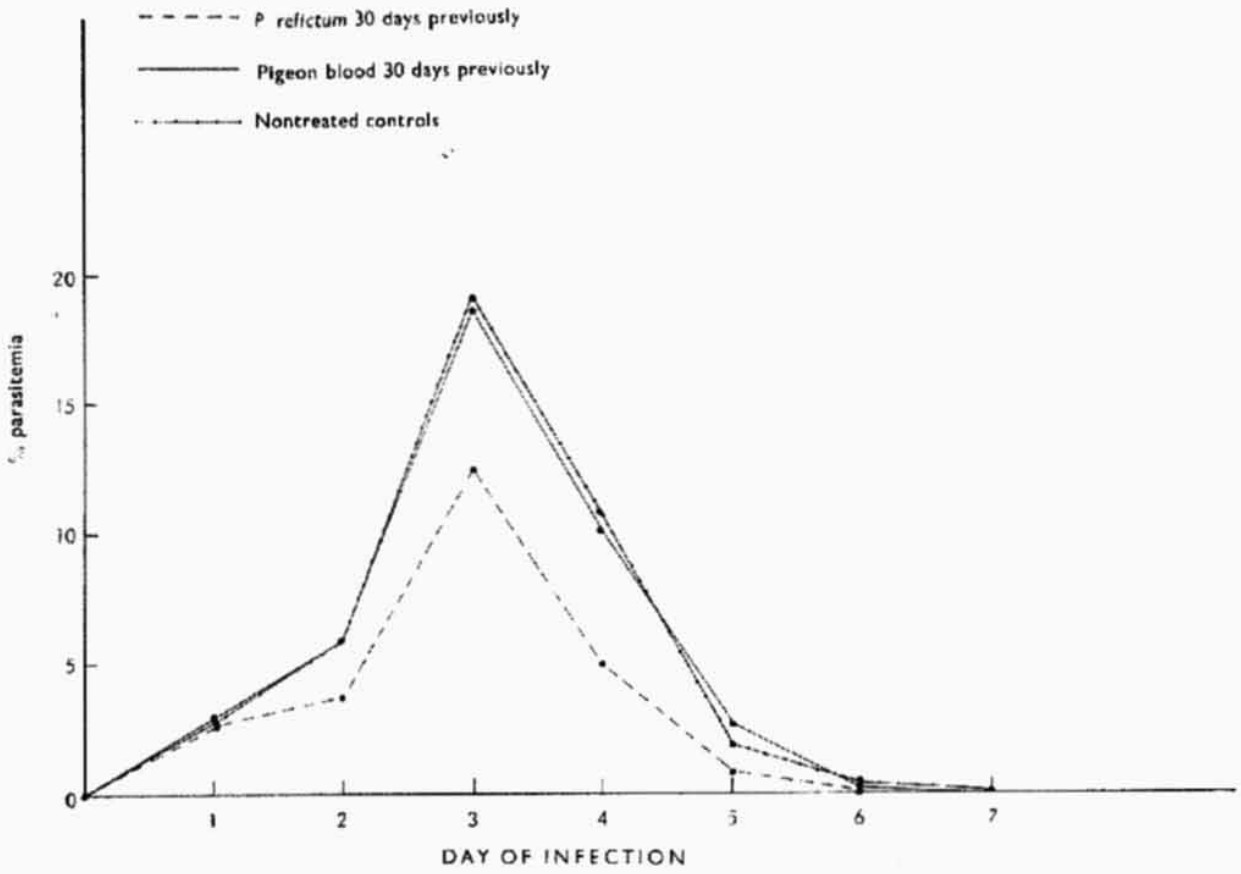

Fig. 8. The course of *P. lophurae* infections in seven-week old White Leghorn chickens. The inoculum contained 10^8 parasitized erythrocytes per kilogram body weight.

period, reaching a peak of parasitemia (mean 15.17 per cent) on day three. Parasites were no longer observed in their blood by day six. The other population was apparently unprotected against *P. lophurae*. Parasites increased rapidly, peaking on day six (mean 42.30 per cent) and were no longer observed by day nine (Fig. 7). Apparently, in some chickens, the previous inoculation of *P. relictum* did not stimulate any protection.

This part of Experiments 3 and 4 included three groups of chickens. Six had been inoculated with *P. relictum* 30 days previously, and six had been injected with clean pigeon blood at the same time. These twelve, and an additional six chickens from the same hatch were inoculated with *P. lophurae* and the courses of infection were followed (Fig. 8). Although infections in all three groups reached their highest level by day three, the mean peak that developed in birds previously infected with *P. relictum* was 12.50 per cent. The other two groups developed mean

parasitemias of 19.1 per cent and 18.7 per cent. No deaths occurred among any of the groups.

The results from this series of experiments demonstrated that *P. relictum* infections were protective against subsequent *P. lophurae* infections. It is important to point out that the protection extended for 7, 14, 21 and 30 days following the initial inoculation with *P. relictum*.

Experiment 5

Six adult pigeons were infected first with *P. lophurae*. Eleven days later, these six and an additional four were infected with pre-crisis *P. relictum*. The courses of both *P. lophurae* and *P. relictum* infections were followed.

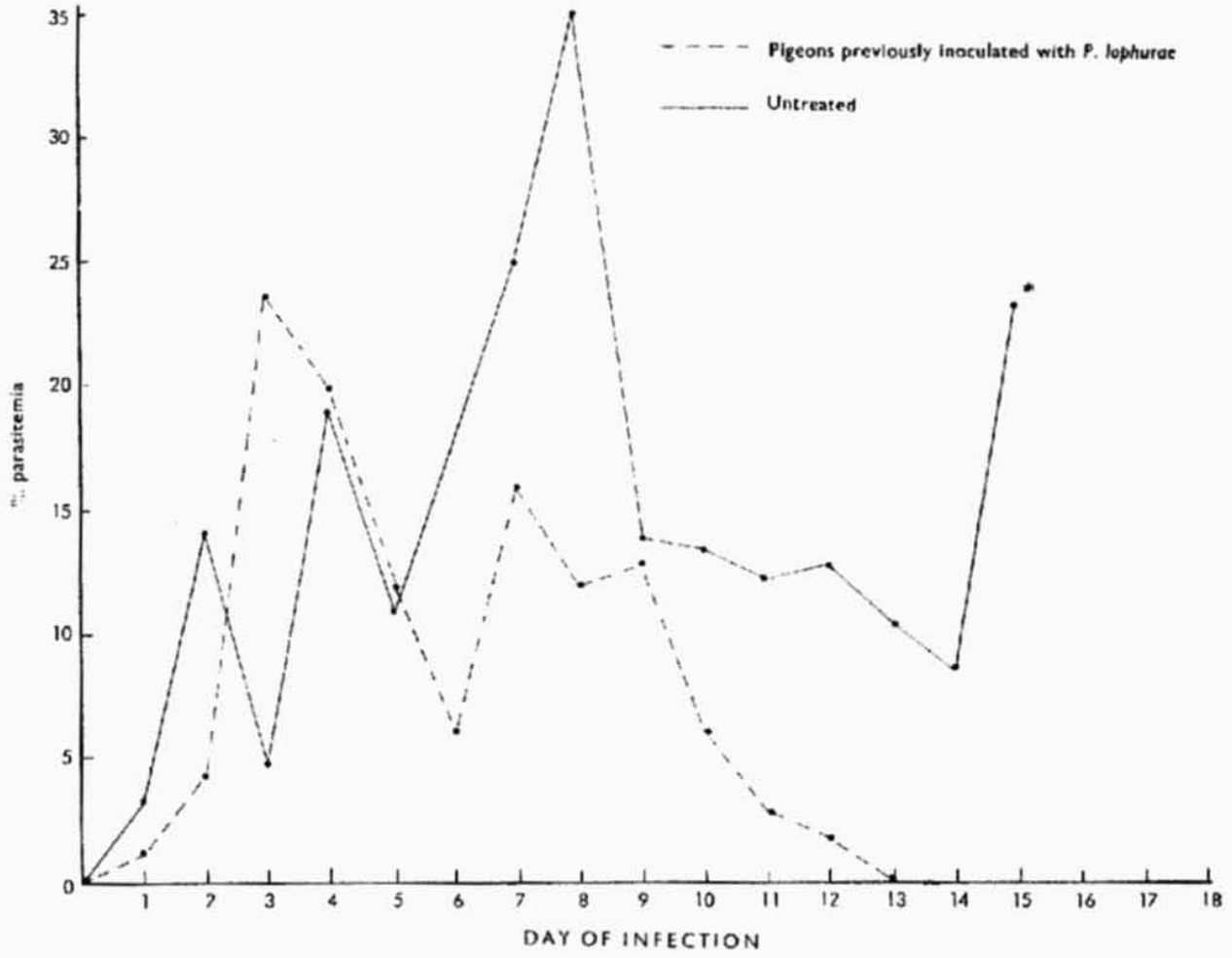

Fig. 9. The course of *P. relictum* infections in adult Modena strain pigeons. The inoculating dose contained 10^8 parasitized erythrocytes per kilogram body weight.

P. lophurae developed mildly in pigeons reaching a peak parasitemia of 0.1 per cent parasitized cells by day four and were no longer detectable in the blood by day seven (Tab. 3). The infection did not develop in one pigeon, i.e., parasites were not observed in blood smears. The course of *P. relictum* infection in both groups was typical, with a peak of parasitemia on days three and four (18.9 per cent in

Table 3. The course of infections of *P. lophurae* in Modena strain pigeons*

	Day of Infection							
	1	2	3	4	5	6	7	8
Parasitemia (%)	0	0	0.075	0.100	0.075	0.050	0	0

controls on day four and 23.4 per cent parasitized cells in *P. lophurae* infected group on the third day), followed by a decrease in parasite numbers. A second peak developed on days seven and eight (15.7 per cent parasitemia in experimental animals and 34.9 per cent in the controls) (Fig. 9). The duration of the infections was 13 days in those pigeons previously inoculated with *P. lophurae*. All the

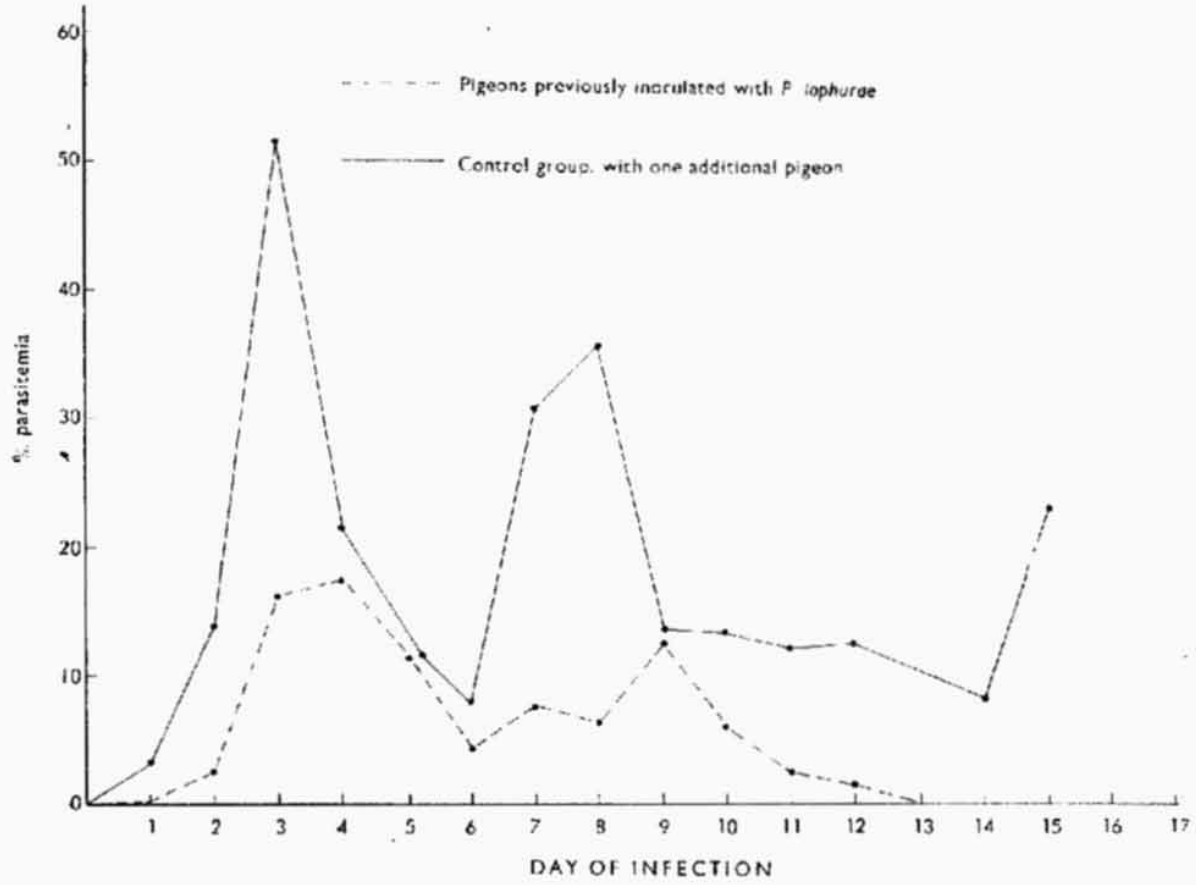

Fig. 10. The course of *P. relictum* infections in Modena strain pigeons. The data are presented with one animal which did not develop a *P. lophurae* infection, in the control group.

P. lophurae non-treated pigeons died by day 15. In the one pigeon, in which *P. lophurae* apparently did not develop, parasite levels were observed comparable to those of the control animals. Apparently this pigeon was not affected by *P. lophurae*, and it was not immunologically sensitized against the inoculation of *P. relictum*. Since it acted like a control, the data were replotted as if it were comparable to the control animals (Fig. 10). Previous exposure with *P. lophurae* seemed to afford definite protection against *P. relictum*. The mortality rates were high in this experiment, and only three of the six birds previously infected with *P. lophurae* survived.

DISCUSSION

There have been few reports on the development of malarial cross-immunity. In fact, numerous investigators have reported a marked absence of any heterologous cross-immunity. MANWELL (1929) failed to note cross-immunity between *P. praecox* and *P. cathemerium*. GINGRICH (1932) observed a similar lack of protective responses between *P. cathemerium* and *P. elongatum* and between *P. relictum* and *P. rouxi*. MANWELL (1934) again described a lack of cross-immunity using *P. vaughani*, *P. cathemerium*, *P. circumflexum*, *P. elongatum* and *P. rouxi*. BOYD and THOMAS (1936) reported no protection between heterologous inoculations of *P. vivax*. MULLIGAN et al. (1940) also reported similar findings.

Where cross-immunity has been described, the reactions have been of varying degrees. GINGRICH (1932) indicated a partial and variable protection between *P. cathemerium* and both *P. relictum* and *P. rouxi*. MANWELL (1938) observed reciprocal immunity between nine species of avian malaria. TALIAFERRO and TALIAFERRO (1945) reported that *P. gallinaceum* conferred a heterologous protection against *P. lophurae*. However, a reciprocal immunity was only slightly effective.

The data obtained during the present study revealed the presence of a reciprocal cross-immunity between *P. lophurae* and *P. relictum*. Chickens first infected with *P. relictum* were protected against subsequent infections of *P. lophurae*. Similarly, pigeons first inoculated with *P. lophurae* were able to combat subsequent *P. relictum* infection more readily. An interesting aspect of this reciprocal protection was that the infections with *P. relictum* in chickens and *P. lophurae* infection in pigeons were extremely mild. Although the duration of these infections was short, they were still capable of stimulating protective mechanisms within the host. This observation appeared to contradict the suggestion by SINTON (1939a, 1939b) that the degree and rate of development of immunity to malaria is dependent upon the amount and duration of antigenic stimulation.

Another explanation for the development of malarial immunity was suggested by REDMOND (1939), who stated that virulence of the parasitic infection was correlated with ability to develop protection. Presumably, the more pathogenic strains, as antigens, conferred their immunity by stimulating immune mechanisms more than less pathogenic strains. Since these different strains may possess common antigens, a cross-immunity may be established. Application of these concepts to the immunity conferred by *P. relictum* upon chickens later infected with *P. lophurae* would suggest that in this case, virulence is not a factor. The normal course of *P. relictum* infections in chickens was extremely mild. The highest level of parasitemia observed was 0.18 per cent. However, the inoculation of *P. relictum* in chickens induced protection to an extent that subsequent infections of *P. lophurae* were reduced for as long as four weeks following the initial infection of *P. relictum*. Therefore, neither duration of infection nor virulence explained the results obtained during this study.

Resistance may be a function of the immunological condition of the original infection. FARMER and BREITENBACH (unpublished) indicated that *P. relictum* developed in chickens only if the inoculating dose was withdrawn from the donor pigeon prior to the crisis of the infection. When the inoculating dose was withdrawn post-crisis and injected into chickens, *P. relictum* failed to develop. The former inoculating dosage conferred immunity, whereas the latter inoculating dosage did not. This suggested that some immunological factor was associated with the erythrocyte-parasite complex. MANWELL (1940) suggested the elaboration of an opsonin to explain protective serum. If an opsonin had been elaborated post-crisis, these "marked" erythrocytes could be removed from circulation more rapidly. In the present study, however, both pre- and post-crisis infections of *P. relictum* were protective. The post-crisis infection remained in the circulation for only 24 hours, yet this exposure was sufficient to stimulate the host's defensive mechanisms since protection was as great as that conferred against *P. lophurae* by pre-crisis infections of *P. relictum*. Apparently, so long as *P. relictum* remained in the circulation for at least 24 hours, whether induced by pre- or post-crisis organisms, it stimulated protection against *P. lophurae*.

Although a reciprocal cross-immunity had been indicated, the results of one part of Experiment 2, in which *P. lophurae* was inoculated 17 days after the original *P. relictum* infection, and one part of experiment 4, in which chickens inoculated with *P. relictum* received *P. lophurae* 21 days following inoculation of *P. relictum*, suggested a decreasing protection with time. However, the majority of the data support the conclusion that protection persisted even after 30 days. It is apparent that in Experiment 5 there were two different groups of birds within the pre-treated animals. Half of the birds developed *P. lophurae* infections, the duration and intensity of which were comparable to infections in controls. When the data obtained from these two groups were separated, it became quite obvious that some of the pre-treated animals were definitely protected. The question remains, however, concerning the reason for the lack of protective stimulation in half of the animals. There is a possibility that in some animals *P. relictum* may not remain in the circulation long enough to stimulate protective mechanisms. The chickens in Experiment 2 (inoculated 17 days previously with *P. relictum*) may also be an example of such a possibility. Unfortunately, the courses of the original *P. relictum* infections in these animals were not followed.

The reciprocal protection induced by *P. lophurae* and *P. relictum* may indicate the presence of common antigens between these two species. HALL (1953) referred to a protozoan or microorganism as an antigenic complex. In his discussion concerning factors involved in acquired resistance, he stated that "One or more similar, or possibly identical, antigens (group antigens) may occur in several strains or in several species. Introduction of an antigenic complex would thus induce the appearance of antibodies corresponding to the antigens of the complex. Antibodies induced by group antigens will react with related micro-organisms which possess such antigens. These types of antigen-antibody reaction are called 'group reactions'

and may form the basis of cross-immunity." It would be reasonable to expect that during the evolution and development of the various species of *Plasmodium* such common antigen groups would be part of closely related species. Immunotaxonomic relationships are, of course, well known in other taxa (LEONE 1965).

It is apparent from the evidence obtained during this investigation that previous infection with *P. relictum* afforded cockerels partial protection against subsequent infections of *P. lophurae*. Furthermore, it was established that this protection can be induced by using inocula from either pre- or post-crisis infections. The specific duration of this protective effect was not determined. However, it persisted for at least 30 days following the initial *P. relictum* inoculation. Furthermore, a reciprocal protective response was suggested, since *P. lophurae* inoculated into pigeons subsequently caused lowered parasitemias of *P. relictum* infections.

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