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Studies on louping-ill virus (Flavivirus group) in wild red grouse (*Lagopus lagopus scoticus*)

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SUMMARY

Studies were made to find evidence of louping-ill virus infection in free-living red grouse and relate this to their breeding success. In areas where ticks were abundant 61 (84 %) adult grouse had antibody to the virus compared with 1 (10 %) in areas where ticks were relatively scarce. Of 162 chicks tested 25 were shown to be viraemic. Infected chicks were of significantly less weight than comparably aged uninfected birds and the probability that they died was much greater than that of uninfected birds. It is concluded that the relatively poor breeding success in areas of high tick numbers was principally due to infection with louping-ill virus. The susceptibility of the red grouse to infection is discussed.

INTRODUCTION

The isolation of louping-ill virus from the brains of red grouse (*Lagopus lagopus scoticus*) found dead or moribund has been reported on several occasions (Williams, Thorburn & Ziffo, 1963; Watt, Brotherston & Campbell, 1963; Reid & Boyce, 1974). In an experimental study of the disease in captive grouse, all infected birds developed clinical signs of depression and weakness and 29/37 died (Reid, 1975*a*). These findings suggested that in areas where louping-ill virus occurs it could be an important cause of mortality in grouse.

Red grouse are scarce and breed poorly on ground where ticks (*Ixodes ricinus*) are numerous (Duncan *et al.*, in the Press). As parasitism by ticks alone cannot account for these low numbers, this effect might be due to the transmission of louping-ill virus. This paper describes studies made to detect virus infection in wild grouse and to compare this with their breeding success.

MATERIALS AND METHODS

Study areas

Five study areas of moorland covered predominantly by heather (*Calluna vulgaris*) were selected near Grantown-on-Spey, Scotland. *I. ricinus* was abundant on three areas designated 'high-tick' (H1, H2 and H3), but relatively scarce on two 'low-tick' areas (L1 and L2) (Duncan *et al.*, in the Press).

Counting and catching grouse

Grouse were studied from June 1 to July 31 in 1974 and from March 15 to July 31 in 1975, as described by Duncan *et al.* (in the Press). Broods were found by setter dogs and the chicks and adults were caught in nets. Adult cocks were also caught during spring in foot snares set around decoy birds. When caught, birds were weighed, aged and marked for individual identification. Blood samples were collected for antibody assay, and from June 1975 onwards also for virus isolation.

Collection of blood samples

Blood samples for virus and antibody assay were collected as described by Reid (1975*a*). For antibody assay, 0·08 ml of blood was collected from the radial vein in a capillary tube containing heparin, and then immediately diluted 1/10 in 0·85 % saline containing 0·5 % bovine albumin. For virus isolation, blood was collected similarly, except that the 1/10 dilution was made in tissue-culture medium.

All diluting fluids were kept on ice in a vacuum flask. Specimens for antibody assay were held at 4 °C until taken to the laboratory, where the cells were sedimented and the supernatant fluid collected and stored at −20 °C until tested. Specimens for detection of virus were transported to the laboratory in a container with solid CO_2, where they were stored at −70 °C.

Testing of samples

Haemagglutination-inhibiting (HI) antibody was tested for as described by Clark & Casals (1958), except that the diluted plasma was extracted with an equal volume of kaolin suspension. Tests were carried out in WHO leucocyte haemagglutination plates. Antigen was prepared from an acetone-ether extract of sucking-mouse brains infected with the standard Moredun isolate of louping-ill virus LI/31, and 4–8 haemagglutination units were used per well.

The virus content of blood was determined using pre-formed monolayers of a pig-kidney cell line (1BRS2 clone 60) in 60 mm plastic Petri dishes as described by Reid & Doherty (1971). Each of the diluted blood specimens was tested for plaque-forming units of virus by thawing it rapidly and inoculating 0·2 ml into one culture, the remainder of the specimen being immediately refrozen. Specimens that produced a confluent cytopathic effect when initially tested were retested in the same way at a dilution estimated to give approximately 100 plaques per culture. All positive samples were further tested using two more culture plates, one of which was also inoculated with 0·2 ml of a 1/100 dilution of sheep antiserum to LI/31.

Table 1. *Haemagglutination-inhibiting antibody to louping-ill virus in red grouse plasma*

	Area														
	H1			H2			H3			L1			L2		
Period	M	F	C	M	F	C	M	F	C	M	F	C	M	F	C
June–July 1974	4/4*	1/1	3/28	—	3/3	2/17	1/1	1/1	9/11	1/2	0/1	0/19	0/2	0/4	0/26
March–May 1975	12/14	—	—	7/11	—	—	7/7	—	—	—	—	—	—	—	—
June–July 1975	3/3	10/10	6/74	1/1	1/4	2/29	4/4	6/9	18/64	—	—	—	—	—	—
Percentage positive	90	100	9	67	57	9	100	70	36	50	0	0	0	0	0

* Number positive/number tested.

M, adult male; F, adult female; C, chick. H1–3 = areas of high tick incidence. L1–2 = areas of low tick incidence.

Table 2. *The reciprocal haemagglutination-inhibiting antibody titres in red grouse plasma*

HI titre	< 20	20	40	80	160	320	640	1280	2560	⩾ 5120
Chick	229	5	7	7	3	7	2	5	3	1
Adult male	9	—	1	3	—	15	6	6	6	3
Adult female	12	—	—	—	—	4	8	5	1	4
Total	250	5	8	10	3	26	16	16	10	8

RESULTS

Detection of antibody

Of the 73 adult birds sampled from the high-tick areas, 84 % had antibody to louping-ill virus, whereas only 1 (11 %) was found positive on the low-tick areas (Table 1). Also, of the 223 chick blood samples from the high-tick areas, 18 % were positive, whereas all 45 samples from the low-tick areas were negative. Titres in positive plasma ranged from 1/20 to 1/5120 (Table 2).

In 1975, birds were sampled only on the three high-tick areas and from most birds blood was collected for both antibody and virus assay. In the first week of chick-catching, 7 (11 %) proved positive for antibody alone, but as 6 chicks were less than 10 days old the antibody in these cases was probably of maternal origin (Table 3). The titres in these 6 plasma samples were between 1/20 and 1/160. The remaining sample had a titre of 1/1280 and came from a chick that was viraemic at the time of sampling. In the second and third weeks of study, approximately 10 % of the chicks had antibody and thereafter about 30 % proved positive.

Detection of virus

Of the 162 samples tested for virus, 15 % were positive (Table 3) and plaque-forming units were identified on both occasions when each positive sample was tested. No plaques developed in any of the plates that had been inoculated with a positive sample and anti-louping-ill serum. The titres of virus detected ranged from $10^{1\cdot48}$ to $10^{6\cdot15}$ plaque-forming units per 0·2 ml of blood (Table 4). The proportion of chicks found to be viraemic increased from the first week of sampling when 9 % were positive, to 37 % in the fourth week; thereafter the proportion of viraemic chicks decreased.

Sequential observations

In June and July 1975, individual broods were caught several times, and presence of virus and antibody was compared with the survival of the chicks. Of the 42 chicks present at the beginning of the study, 3 were still present at the end of July, 4 were known to have died and it was assumed that the 35 other missing chicks had also died (Table 5). The assumption about death rested on a tagged chick being missing from a brood and never again observed on occasions when the other members of that brood were seen. The observer presumed that all chicks had died when a tagged hen previously observed with chicks, was seen with none.

Of the 4 chicks known to have died, 2 were found dead, one was discovered

Table 3. *Detection of antibody and virus in the plasma of red grouse chicks in* 1975

	Week of observation											
	1		2		3		4		5		6	
Area	V	Ab	V	Ab	V	Ab	V	Ab	V	Ab	V	Ab
H1	0/24*	2/30	1/19	1/19	4/19	1/19	2/2	1/2	0/3	1/3	0/1	0/1
H2	1/16	0/16	1/5	1/5	1/4	0/4	1/4	1/4	—	—	—	—
H3	4/16	5/15	2/10	2/10	0/5	1/5	4/13	4/13	2/13	4/12	2/8	2/8
Percentage positive	8·9	11·5	11·8	11·8	17·9	7·1	36·9	31·6	12·5	31·3	22·2	22·2

* Number positive/number tested.
V, virus, Ab, antibody.

Table 4. *The titres of louping-ill virus detected in the plasma of red grouse chicks, expressed as plaque-forming units per 0·2 ml of blood*

Titre	10^1	10^2	10^3	10^4	10^5	10^6
Number of chicks	3	3	6	3	9	1
Range of titre	$3{\cdot}0–5{\cdot}0 \times 10^1$	$5{\cdot}8–7{\cdot}0 \times 10^2$	$1{\cdot}1–3{\cdot}3 \times 10^3$	$2{\cdot}1–8{\cdot}5 \times 10^4$	$1{\cdot}1–7{\cdot}6 \times 10^5$	$1{\cdot}4 \times 10^6$

Table 5. *The decline in size and identification of infection in sequentially observed broods of red grouse chicks*

	Week of study					
Brood no.	1	2	3	4	5	6
1:1	0/3+0/5 (8) (8)	0/3 (5)	3/4 (4)	(0)	(0)	(0)
1:2	0/3 (6)	2/6 (6)	2/2 (2)	NT	1/1 (1)	1/1 (1)
1:3	0/5 (5)	0/4 (4)	0/4 (4)	NT	0/1 (1)	0/1 (1)
1:4	NT (5)	0/5 (5)	0/4+0/2 (5) (3)	2/2 (2)	(0)	(0)
2:1	0/4 (7)	1/4 (6)	1/3 (3)	1/2+1/1 (2) (1)	(0)	(0)
3:1	2/4 (4)	3/4 (4)	1/1 (1)	(0)	(0)	(0)
3:2	NT	NT	0/4 (7)	3/5 (7)	2/5+1/2 (5) (2)	1/1 (1)
Total	2/24	6/26	7/24	7/10	4/9	2/3
Mean brood	6·1	5·0	3·6	2·0	1·1	0·4

Number of chicks positive for virus and or antibody/number tested. (Number of chicks observed.)

Two observations of the same brood made during the week.

NT, not tested.

moribund and was killed, and one was accidentally killed during capture. One of the dead chicks, from brood 2:1, was found decomposed 9 days after it had been sampled and shown to have an antibody titre of 1/1280. The other dead bird came from brood 3:1 and had been shown to be viraemic 5 days before the freshly dead carcass was discovered. Virus was recovered from the brain of this chick at a titre of $10^{5{\cdot}15}$ p.f.u./0·2 g, and from the brain of the moribund chick at $10^{4{\cdot}59}$p.f.u./0·2 g.

During the period of study, 90 blood samples for virus isolation and 95 for antibody detection were collected from the 42 chicks. In this way 21 chicks were shown to have been infected with louping-ill virus. Only 4 of these chicks were again observed alive following identification of infection and the other 17 were assumed to have died. By contrast, no evidence of infection was found in 61 samples, and 45 individual chick observations were subsequently made of the chicks from which these samples were collected. Thus, following the initial detection of infection

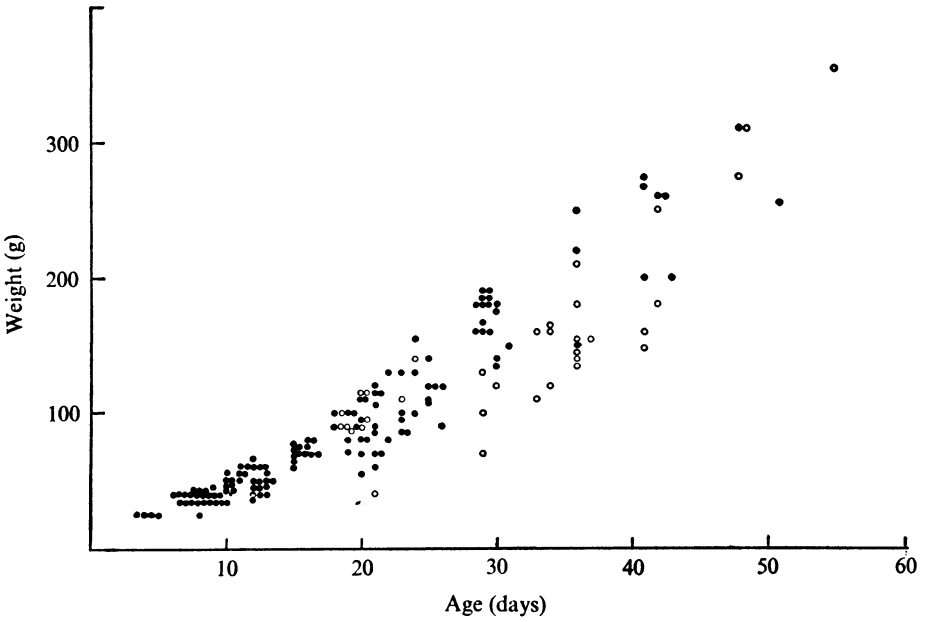

Fig. 1. The relation of weight of infected (○) and uninfected (●) red grouse chicks to age.

Table 6. *The breeding success of red grouse on the five study areas*

	Area				
Year	H1	H2	H3	L1	L2
1974	6 (0·86)	5 (1·00)	1 (0·17)	61 (5·08)	96 (6·00)
1975	6 (0·45)	0 (0·00)	9 (1·13)	51 (5·10)	46 (5·75)
Total	11 (0·61)	5 (0·50)	10 (0·71)	112 (5·09)	142 (5·92)

Number of chicks, and in parentheses the ratio of number of chicks to number of hens. H1–3 = area of high tick incidence. L1–2 = area of low tick incidence.

in a chick, the probability that the chick would never be seen again was significantly greater than for an uninfected chick to be seen on subsequent visits ($P < 0{\cdot}001$).

Weight of chicks

The weight of all chicks increased with age (Fig. 1). However, infected chicks did tend to weigh less than uninfected birds of the same age and an analysis of covariance showed this difference to be significant ($P < 0{\cdot}01$).

Breeding success

The breeding success of grouse on each area was assessed from the ratio of young birds to adult hens in the last week of July. In both years, grouse on the three high-tick areas bred less successfully than on the low-tick areas (Table 6).

DISCUSSION

The HI antibody detected in this study was entirely due to infection with louping-ill virus as this is the only Flavivirus known to be present in the British Isles. Furthermore, the titres detected were generally high, 75 % being > 1/320. All virus identified in the plasma of grouse formed plaques identical with those formed by standard isolates of louping-ill virus, and in each case complete neutralization was achieved using immune serum raised in sheep to the LI/31 isolate. The 25 viraemias detected were therefore due to louping-ill virus.

On the three high-tick areas, 84 % of adult grouse had antibody to louping-ill virus. In addition a high proportion of chicks became infected in their first eight weeks of life and of these only a small number survived. The average number of chicks per brood at the end of July was 0·6 compared with 5·5 on the low-tick areas. As the size of broods when hatched was similar on all study areas, the small number of chicks reared on high-tick areas was due to death occurring during June and July.

Chicks infected with louping-ill virus weighed significantly less than uninfected chicks of the same age, which suggests that infection was associated with either loss of weight or a check in live-weight gain. In addition, infected chicks were more likely to die between observations than uninfected chicks. As birds 4–7 months old survived for a mean of only 5·9 days following peripheral inoculation with louping-ill virus (Reid, 1975*a*), some of the 20 chicks dying between observations and showing no evidence of infection probably did become infected and die in the interval between observations. Furthermore, of the 4 chicks found to have been infected and subsequently observed, only one was still present at the end of July; the remaining 3 may have died from louping-ill virus infection, as deaths in experimentally-infected birds occurred up to 13 days after inoculation (Reid, 1975*a*). More ticks were found on grouse on the high-tick areas and this may have contributed to the mortality. However Duncan *et al.* (in the Press) found that tick burdens alone could not explain the mortality observed. Factors other than louping-ill virus infection may have contributed to the poor breeding success in the high-tick areas, but the evidence suggests that infection was the principal cause. This idea is given further support from evidence involving the 2 dead and 1 moribund chicks that were found. The 2 dead chicks became infected shortly before death and in one case virus was isolated from the brain. In addition, virus was recovered from the brain of the chick found moribund, and Dr D. Buxton (personal communication) found that this brain had histological lesions indistinguishable from those developing after experimental infection with virus (Buxton & Reid, 1975).

For a stable population to be maintained, the numbers of grouse available for recruitment must exceed the mortality rate. As a result of studies on many different areas, Jenkins, Watson & Miller (1967) showed that the numbers of breeding birds in spring tended to decline after years when the breeding success fell below about 2–3 young reared per hen. As only 0·6 chicks were reared per hen on the areas where louping-ill was prevalent during the present study, one would expect the breeding stock of grouse there to decline.

Beasley, Campbell & Reid (in the Press) found that virus was transmitted to *I. ricinus* nymphs and larvae only when viraemias exceeded 10^3 and 10^4 p.f.u. per 0·2 ml respectively. In the present study 76 % of the viraemias detected in grouse exceeded 10^3. Red grouse can therefore act as amplifier hosts for louping-ill virus, but this amplification may be only temporary if a grouse population cannot persist for long in an active focus of louping-ill. The authors are aware of only one other report of disease occurring in wild species following infection with a Flavivirus: this was the mortality of monkeys following infection with Kyasanur Forest disease virus in India (Boshell, 1969). This infection came into the monkey (*Presbytis entellus* and *Macaca radiata*) population after grazing cattle, taken into the monkey's forest habitat, had greatly increased the number of hosts for the tick *Haemophysalis spinigera*. Similarly on the heather moorlands of Scotland, the habitat of the red grouse, the number of hosts for *I. ricinus* may have been much smaller before the development of the sheep industry on these moors during the 19th century (Reid, 1975*b*). In both India and Scotland the introduction of large numbers of domestic stock into a new habitat resulted in many more hosts being available for ticks. Thus the virus may have been introduced into a wild species which were highly susceptible because they had probably had little or no previous contact with the virus.

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