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13. ABSTRACT *(Maximum 200 words)*

A total of 3524 rodents collected in a HFRS endemic area of Sweden were tested for Puumala (PUU) virus infection (both viral antigen in lungs and specific antibodies in serum) by ELISA. The material included 2493 Clethrionomys glareolus, 620 C. rufocanus, 367 Microtus agrestis, 39 Myopus Schisticolor and 5 Apodemus flavicollis. C. glareolus was found to be the most important vector of PUU virus. Infection rate did correlate with population density over time but with a time lag of one year.
In addition monoclonal antibodies to PUU virus were generated and used for characterisation of virus and development of new diagnostic techniques. Two epidemiological studies, one in Bashkirtostan (Russia) and one among military personnel in Sweden were also performed. Sequele post HFRS in man were investigated.

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January 28 1993 B Niklasson
Date Bo Niklasson Principal Investigator

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BACKGROUND

Between 1951 and 1954, more than 3000 United Nations troops stationed in Korea developed a disease not previously recognized by western physicians. The illness, which was characterized by fever, headache, abdominal pain, renal dysfunction and various hemorrhagic manifestations, became known as Korean hemorrhagic fever. It soon became clear that clinically identical or closely related diseases occurred in large parts of Asia and Europe. The name hemorrhagic fever with renal syndrome (HFRS) was suggested by Carlton Gajdusek and is now widely accepted.

About 150.000 patients are hospitalized each year with HFRS. Nephropathia epidemica (NE), one form of HFRS, causes considerable human morbidity in Scandinavia, Finland and western Russia. The etiological agent of NE, Puumala virus (PUU), was isolated in 1983 from a bank vole (Clethrionomys glareolus) in Sweden (1). As part of previous research performed with grants from US Army a nation-wide study of NE was conducted in Sweden, identifying the major endemic area of the disease (2). This study indicated that the bank vole is the most important vector of PUU virus. The basic biology of this species is thus directly related to the incidence of NE disease in humans. The aim of the present study was to generate data on ecology of bank voles and the dynamic of PUU infection in the vole population with the long term gaol to use this data for remote sensing. The present study does only cover a detailed study of PUU infection in a highly endemic area of Sweden while studies using eg satellite images are to be performed separately.

In addition to the primary scope of this work additional studies on HFRS have been performed within the HFRS project. These studies have mainly been funded by FOA, SBL and the Swedish research council but have been carried out in collaboration with USAMRIID and Col LeDuc. The results of these additional studies are given in summary in this final report and in more detail in the enclosed publication and manuscripts.

HFRS INFECTION RATE AND POPULATION DYNAMICS

Animals

Small rodents have been collected at 16 different specified sites in a highly endemic area (Västerbotten (AC) county 64°N, 20°E) of Sweden twice every year during the same weeks in May and September since 1971. At each trapping occasion 2840 traps have been placed during 3 consecutive days (8520 "trap-nights"). Animals were trapped in snap-traps, collected twice a day and stored at -20°C until processed in the laboratory. Small rodents collected between 1979 and 1986 were included in the present study.
Species, weight, date and location of trapping were available. Weight was recorded since it corresponds well with age of the animal. Two punch-necropsies of 2,0 mm (in diameter) were taken through the thorax of the animal on each side. The 4 necropsies were grinded in 2 ml of PBS using mortar and pestle. Lung tissue from all animals were investigated for both presence of PUU virus antibody and antigen in two separate ELISA.

Immune reagents used in antibody and antigen ELISA

The preparations of PUU virus antigen, negative control antigen and rabbit (Chinchilla breed) immune serum are described elsewhere (3). Convalescent sera of 2 patients with very high antibody titers to PUU virus were used. Human and rabbit immunoglobulin to PUU virus were prepared by ammonium sulphate precipitation.

Detection of PUU virus specific IgG antibodies by ELISA

A sandwich ELISA was employed as follows. Rabbit anti-PUU virus immunoglobulin diluted 1:400 in coating buffer (0,05 M sodium carbonate, pH 9,5 to 9,7) was absorbed to 96 well polystyrene microtiter plates (Cooke M 29 AR Dynatech laboratories) at 37°C for 1 h, followed by virus cell lysate antigen diluted 1:4 in ELISA buffer (phosphate-buffered saline without Mg^{++} and Ca^{++} and with 0,05% tween 20 and 0,5% bovine serum albumin), grinded lung specimen (diluted 1:2 in ELISA buffer for 1 h at 37°C, and goat anti-mouse IgG conjugated with alkaline phosphatase diluted 1:600 in ELISA buffer at 37°C for 1 h. P-nitrophenol-phosphate (Sigma) diluted in diethanolamine buffer (1 M diethanolamine pH 9.8, 0.5 mM $MgCl_2$) was used as the substrate. Washing between each step was done 6 times in washing buffer (saline with 0.05% Tween 20). The

reaction was read after 30 min at room temperature in a spectrophotometer at 405 nm and expressed as optical density (OD). Optimal dilutions of all reagents used in the ELISA were determined by box titration. All specimens were tested in duplicate with antigen and negative control antigen. The OD was calculated as the average OD with antigen minus the average OD with negative control antigen. To adjust for plate-to-plate and test-to-test variations in the assay, a positive control serum was included on all plates. This control had an OD of 0.700 (in the linear interval of this IgG ELISA). If the positive control serum had an OD between 0.500 and 0.900 the plate was accepted: however, all OD values on that plate were multiplied by a factor as to set the positive control value at 0.700.
The boarder between positive and negative were determined as the mean of 100 known negative specimens plus 3 SD giving a cut off limit of OD 0.180.

Detection of PUU virus specific antigen by ELISA

The test format for PUU virus antigen are described in a previous publication (4). In short, a high titer human (serum 1) anti PUU Ig diluted 1:400 in coating buffer was absorbed to 96 well polystyrene microtiter plates (Cooke M 29 AR Dynatech laboratories) at 37°C for 1 h, followed by grinded lung specimen (diluted 1:2 in ELISA at 37°C for 1 h and peroxidase labelled high titer human anti PUU (serum 2) and TMB substrate. Washing between each step was done 6 times in washing buffer (saline with 0.05% Tween 20). The reaction were stopped after 5 min by H_2SO_4. The optical density was read at 450 nm at room temperature. Optimal dilutions of all reagents used in the antigen ELISA and adjustment for plate-to-plate and test-to-test variations in the assay were calculated as described above for IgG ELISA was used.
The boarder between positive and negative were determined as the mean of 100 known negative control specimen plus 3 SD giving a cut off limit of OD 0.070.

RESULTS.

All results (raw-data) computerized using dBaseIII plus format have been sent to Dr James LeDuc, DAD/USAMRIID.

A total of 3524 rodents were tested including 2493 C glareolus, 620 C rufocanus, 367 Microtus agrestis, 39 Myopus Schisticolor and 5 Apodemus flavicollis. The proportion of antigen and antibody positive animal are shown in table 1. All sorex species trapped (n=377) were excluded from the study. Infection rate was defined as animals being either antibody positive or antigen positive or both.

Clethrionomys glareolus

C glareolus was found to be the dominating species with 64% of the rodents trapped. Figure 1 shows the number of C glareolus in different weight groups as well as the proportion of PUU virus infected individuals. Both antibody and antigen positive prevalence increased with weight/age. Antigen decreased in animals with only antigen with increasing weight/age. The infection rate was more than 50% in the highest weight/age groups.

The number of rodent trapped between 1979 and 1987, expressed as numbers of individuals per 100 trap nights (trap index) as well as the average weight/age of the animals is seen in figure 2. Two periods with high trap index was noted; 1981-1982 and 1985. In the analysis of vole abundance over time we defined two cycles as 1979-1982 (cycle 1) and 1983-1985 (cycle 2). There was a seasonal variation with greater number of animals in the fall as compared with the spring. The weight/age on the other hand showed a opposite pattern with higher mean weight/age in the spring as compared with the fall.

Trap index in relation to the prevalence of infected animals (either positive for antibody or antigen or both) is seen in figure 3. The infection rate was density dependent with a time-lag of approximately 1 year. The correlation between trapping index and infection rate by regression analysis gave a correlation coefficient (r) of 0.03, $p>0.1$. The correlation between the infection rate and previous years trapping index by regression analysis gave a correlation coefficient (r) of 0.79, $p<0.001$. Areas were sorted based on bank vole abundance during the first cycle in figure 4 A. The total number of bank voles trapped at the

16 locations were compared for cycle 1 and 2. There was a significant correlation between the trap index at the different locations between the two cycles ($r=0,82$ $p<0.001$). However, the infection rate did not correlate with trap index for the different areas in neither cycle 1 nor in cycle 2 (figure 4 B $p>0.1$).

Clethrionomys rufocanus and Microtus agrestis

Figure 5 and 6 shows the number of C rufocanus and M agrestis in different weight groups as well as the proportion of PUU virus infected individuals.

C rufocanus showed a increase of infection rate by weight/age with a relative high frequency of animals with only antigen in the lower weight/age groups while positive animals in the highest weight/age groups only had antibodies. In general antigen positive C rufocanus showed low OD readings (mean OD of the positives=139) as compared to C glareolus (mean OD of the positives=322). The infection rate in C rufocanus was 15-25% in the highest weight/age groups.

M agrestis was low in absolute numbers and showed a relative low infection rate. Weight/age showed a normal distribution similar to C glareolus and C rufocanus. However, no increase in infection rate by weight/age could be detected. The mean OD of antigen positive animals were only 120.

DISCUSSION

C. glareolus is the most abundant small mammal in Sweden and is commonly found from the southern tip of the country northward to near the arctic circle. Although only one subspecies of C. glareolus is recognized throughout the Swedish mainland, a gradient exists in population stability. Populations of C. glareolus in the south of Sweden are non-cyclic, whereas those in the north fluctuate on a three- to four-year cycle of abundance. During peak populations, voles may be 1.000 times more abundant than immediately after population crashes. The number of human cases in the endemic area correlates with the fluctuation of voles (5). There is a boundary between the cyclic population in the north and the more or less non-cyclic population in the south. This boundary corresponds to the limes norrlandicus, a bio-geographical line running from 59°N on the west coast of Sweden to 61°N on the eastern shore. This line separates the northern boreal (or taiga) zone from the southern boreal-nemoral zone. The limes norrlandicus also appears to demarcate the southern distribution boundary of NE in humans and of PUU virus in voles. Antibody prevalence as well as disease incidence in humans increase to the north of the limes norrlandicus, reaches a peak in a region near the 64°N parallel and then decrease in the far north. Infection-rates in C. glareolus follows the same pattern. Since C. glareolus is suggested to be the most important vector of PUU virus the ecology and biology of this vector is therefore the key for in-depth understanding of the epidemiology of the disease. The aim of the present study was to map infection rate in small rodents over time in a well defined endemic area of Sweden.

In the present study we used detectable antibodies or antigen as a criteria of animals being infected. Previous studies by Lee and coworkers have established that the mouse reservoir of Hantaan virus, a related virus, gets chronically infected without any notable disease but with continuous shedding of virus throughout the animals entire life. Most previous studies among hantavirus infected laboratory animals support the theory that the virus is infectious by aerosol both between animals and between animals and humans. PUU virus is difficult to grow in cellculture and there is no assay to measure infectious virus in animal tissues or

secretions. The question if significant shedding of virus is limited in time or if it continues for life once the animal gets infected is teherfor not fully answered.

However, despite this the data in the present study implicate C. glareolus as the most important vector of PUU virus. Both the abundance and the infection rate is higher for this species as compared to C. rufocanus and M. agrestis.

A great variation between vole abundance in different locations were noted. Areas with low trapping index during the first cycle showed a low index the second cycle. While there were a correlation between trapping index of C. glareolus and C. rufocanus for different locations, M. agrestis showed no correlation with either of these two species. The infection rate in C. rufocanus was approximately half of that in C. glareolus. M. agrestis showed a very low infection rate.

Previous studies have found a seasonal variation in the trapping index as well as a variation in trap index between different year with a periodicity of 3-4 years. Although the present study only cover 8 years a cyclic pattern in bank vole abundance can be noted. The spring trap index is lower than the fall trap index. While the fall follow a full breeding season the spring trap index measures the number of animals that survived the winter. Animals in the spring is therefore generally older (increased weight). Infection rate gives a similar pattern with inter year variation with higher infection rate in the spring as compared with the autumn.

The continuous increase of infection rate with increasing weight/age clearly show that horizontal infection is of great importance in C. glareolus. It can be expected that horizontal spread of infection increase with high population density. However, the present study showed no such correlation between infection rate and trap index for the different locations. In contrast, infection rate did correlate with population density in the whole area over time but with a time lag of one year. This means that infection rate depend on the vole abundance during the previous season.

All serologically confirmed NE cases have been recorded since 1985. Confirmed human cases were compared to the trap index in Västerbotten (animals collected as described in material and

methods) between 1985 and 1990. Although the time period was to short to show any significant correlation between trap index and human cases, it was noted that the correlation coefficient increased when human cases were compared with the trap index of the previous year suggesting a time lag phenomenon as seen with infection rate (6).

Another important question not yet answered is if PUU virus have any negative effect on its veretebrate host. Since such high proportion of the C. glareolus get infected the question wether the virus affect the animal in terms of expected life span or reproduction is of major importance in terms of population dynamics.

ADDITIONAL STUDIES

1. An epidemiological study of hemorrhagic fever with renal syndrome in Bashkirtostan (Russia) and Sweden (6).

Incidence and antibody prevalence of hemorrhagic fever with renal syndrome (HFRS) in Bashkirtostan (European part of Russia) and northern Sweden was compared with the abundance of Clethrionomys glareolus (bank voles) in the two areas. In Bashkirtostan 10% of the women and 15% of the men were found to be antibody positive. The corresponding figures for Sweden were 8% and 16% for women and men, respectively. The annual incidence in Bashkirtostan was 50 cases per 100,000 inhabitants with a male:female ratio of 4.7:1. The incidence in the endemic area of Sweden was 7 per 100,000 inhabitants with a male:female ratio of 1.8:1. A similar age distribution of cases with a peak in the middle age groups, especially in men, was found in both Bashkirtostan and Sweden. The incidence of HFRS in humans and abundance of bank voles varied over time in both Bashkirtostan and Sweden but the study failed to find any significant correlation between the two variables. The study showed that HFRS causes significant human morbidity in the areas studied but that both incidence and possibly also bank vole abundance was higher in Bashkirtostan than in northern Sweden.

2. A study of nephropathia epidemica among military personnel in Sweden (7).

The incidence of nephropathia epidemica among military troops operating in an endemic area of Sweden was investigated. A total of 705 soldiers (age 18-25) involved in field training in three different endemic counties were bled twice with a 6 month interval. Three individuals seroconverted when tested for Puumala virus antibodies by IgG ELISA. Symptoms typical of nephropathia epidemica were not recorded in any of the patients. However mild febrile episodes were recorded in 2 of the 3 individuals. One serologically confirmed soldier with typical nephropathia epidemica symptoms was found in another group of 12.000 troops. This soldier fell ill 3 weeks following a 7 day long field exercise. The present study indicates that military populations are at considerably greater risk to contract nephropathia epidemica as compared to the entire population residing in the same area.

3. Monoclonal antibodies specific for Puumala virus produced by bank vole-mouse heterohybridomas (8).

Nephropathia epidemica, caused by Puumala (PUU) virus, is a milder form of hemorrhagic fever with renal syndrome. Several viruses serologically related or identical to PUU virus cause significant human morbidity in Europe. The lack of specific monoclonal antibodies (MAbs) has hampered more detailed studies of these viruses. Attempts to produce mouse MAbs against PUU virus have been unsuccessful, probably due to absent or low immunological responses in laboratory mice. To overcome this problem we infected the natural host, bank vole (Clethrionomys glareolus), with PUU virus and obtained activated specific B-lymphocytes for the hybridoma production. Bank vole spleen cells were fused with the mouse myeloma cell line SP2/0 and eight PUU-specific monoclonal heterohybridomas were selected for further characterisation. The hybridomas have now been grown for more than eight months and the majority have persisted as stable monoclonal cell lines. Two of the eight hybridomas showed to be less stable and were recloned once to maintain unaltered antibody production. The bank vole MAbs were all highly specific against the nucleocapside protein of PUU virus and no cross-reactions against other proteins were seen. The MAbs have been successfully used in several assays including immunoprecipitation, immunofluorescence and immunoblotting with methods and reagents developed for mouse MAbs. They all have characteristics very similar to mouse IgG MAbs which make them easy to handle and powerful tools for the investigation of viruses associated with hemorrhagic fever with renal syndrome.

4. Hemorrhagic fever with renal syndrome; a study of sequelae following nephropathia epidemica (9).

Sequelae following Nephropathia epidemica (NE) was investigated. 792 individuals living in an endemic area of Sweden were tested for presence of Puumala virus specific IgG antibodies in serum as well as several parameters linked with renal dysfunction and cardiovascular disease (systolic and diastolic blood pressure, ECG, BMI, total cholesterol and serum-creatinine). No difference were seen in any of the variables tested between PUU virus antibody positive and negative individuals. In addition the blood

pressure were measured in 62 serologically confirmed patients diagnosed with clinical NE 2-6 years previously. Only one patient with moderate hypertension was found. In conclusion, the data presented failed to show any evidence of sequelae of clinical significance following NE infection.

5. Antibodies to Puumala virus in humans determined by neutralization test (10).
An assay for detection of neutralizing antibodies to Puumala virus using 96-well microtiter plates (NT-ELISA) was developed and evaluated. The test proved to have similar sensitivity and specificity as an IgG ELISA and indirect immunofluorescence test, when screening 187 sera (with an antibody prevalence rate of 19%) from normal populations in an endemic area of Nephropathia epidemica (NE) in Sweden. NE-patients monitored for 2 years had neutralizing antibodies in early sera collected 1-4 days after the onset of disease with a continuous increase in neutralizing antibodies with time. Furthermore, high titers of neutralizing antibodies were detected 10-20 years post-infection. This neutralization assay was also evaluated as a screening method in the production of monoclonal antibodies. The format of the NT-ELISA makes it feasible to screen a large number of specimens with results similar to the standard plaque or focus-reduction neutralization tests.

6. Hemorrhagic fever with renal syndrome: Evaluation of ELISA for detection of Puumala virus specific IgG and IgM (11).
An IgM and IgG ELISA to Puumala virus were evaluated using sera from patients with hemorrhagic fever with renal syndrome (HFRS) from different geographical regions; Sweden, Denmark, Norway, Belgium and the European USSR.
The IgM ELISA proved useful in the diagnosis of HFRS in patients from all of the regions mentioned above. Specific IgM could be detected as early as day 1 post onset of disease and patients remained IgM positive for several months. Specific IgG ELISA antibodies could also frequently be detected in the acute sera and acute - convalescent serum pairs often failed to show a significant titer rise or a significant increase in optical density (OD) values. This limits the use of IgG ELISA for patient

diagnosis. Sera collected 2 years after the infection revealed higher IgG ELISA OD readings than convalescent sera and very high values was still detectable 10 to 20 years post infection. The IgG ELISA is therefore useful for testing of immunity and seroepidemiological studies.
Acute and convalescent sera from HFRS patients in Korea and the Asian USSR showed no or very weak reactivity by the Puumala virus IgG and IgM ELISA. These results are consistent with the "one way cross" earlier described.

7. Comparison of clinical course in Sweden and in the western Soviet Union (12).
The severity of the clinical course in 355 Swedish and 603 Soviet patients with serologically confirmed hemorrhagic fever with renal syndrome (HFRS) was compared retrospectively. The overall frequency of hemorrhagic manifestations including also benign bleeding such as ecchymoses was higher among Soviet than among Swedish patients (37% vs. 10%). However, severe hemorrhagic complications (gastrointestinal and renal tract bleeding) were equally common in both countries. There were 2 fatal Soviet cases, whereas none of the Swedish patients died. We conclude that the clinical presentation of HFRS in Sweden and in the Western Soviet Union shows many similarities but there seems to be a higher frequency of life threatening complications among the patients in the Western USSR.

8. Comparison of European isolates of viruses causing hemorrhagic fever with renal syndrome by a neutralization test (13).
Different virus isolates causing hemorrhagic fever with renal syndrome (HFRS) were compared using a neutralization test. Patient convalescent sera and antisera prepared in rabbits were used to compare Puumala related hantavirus isolates from Finland, Sweden, Belgium and the USSR. The majority of European isolates were indistinguishable from each other using both homologous rabbit antisera and patient convalescent sera. The European isolates of HFRS were also compared with prototype Hantaan (the etiologic agent of Korean hemorrhagic fever). The one way cross reaction between the Hantaan and Puumala viruses earlier described using human convalescent sera tested by indirect immunofluorescence and

immunoprecipitation was also seen by the neutralization test.

9. Bank vole monoclonal antibodies against the Puumala virus envelope glycoproteins; identification of epitopes involved in virus neutralization (14).

Bank vole (Clethrionomys glareolus) monoclonal antibodies (MAbs) against the two envelope glycoproteins (G1 and G2) of the Puumala (PUU) virus were generated and characterized. Analyses of the MAbs antigen and epitope specificities showed non-overlapping reactivities of one anti-G1 and two anti-G2 MAbs. A significant neutralizing activity was shown by the anti-G1 and one of the anti-G2 MAbs, suggesting the existence of at least one neutralizing domain on each of the two glycoproteins. The two neutralizing MAbs reacted with eight PUU-related (serotype 3) virus strains, but did not react with Hantaan, Seoul, or Prospect Hill viruses in an immunofluorescence assay, indicating reactivity with epitopes unique for PUU virus. The non neutralizing anti-G2 MAb also reacted with Seoul virus, revealing the presence of a conserved G2-epitope common for PUU, and Seoul viruses, not involved in neutralization. Competitive binding of the MAbs and sera from nephropathia epidemica patients indicated that the defined neutralizing and non-neutralizing epitopes of the glycoproteins were immunodominant also in humans. In another experiment, magnetic beads coated with two MAbs were bound with the virus glycoproteins and used for selective enrichment of cells secreting anti-glycoprotein antibodies.

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Figure 1. The number of C. glareolus in different weight groups as well as the proportion of PUU virus infected individuals.

Figure 2. The number of rodent trapped between 1979 and 1987, expressed as numbers of individuals per 100 trap nights (trap index) as well as the average weight/age of the animals. F=fall S=spring

Figure 3. Trap index in relation to the prevalence of infected animals (either positive for antibody or antigen or both).

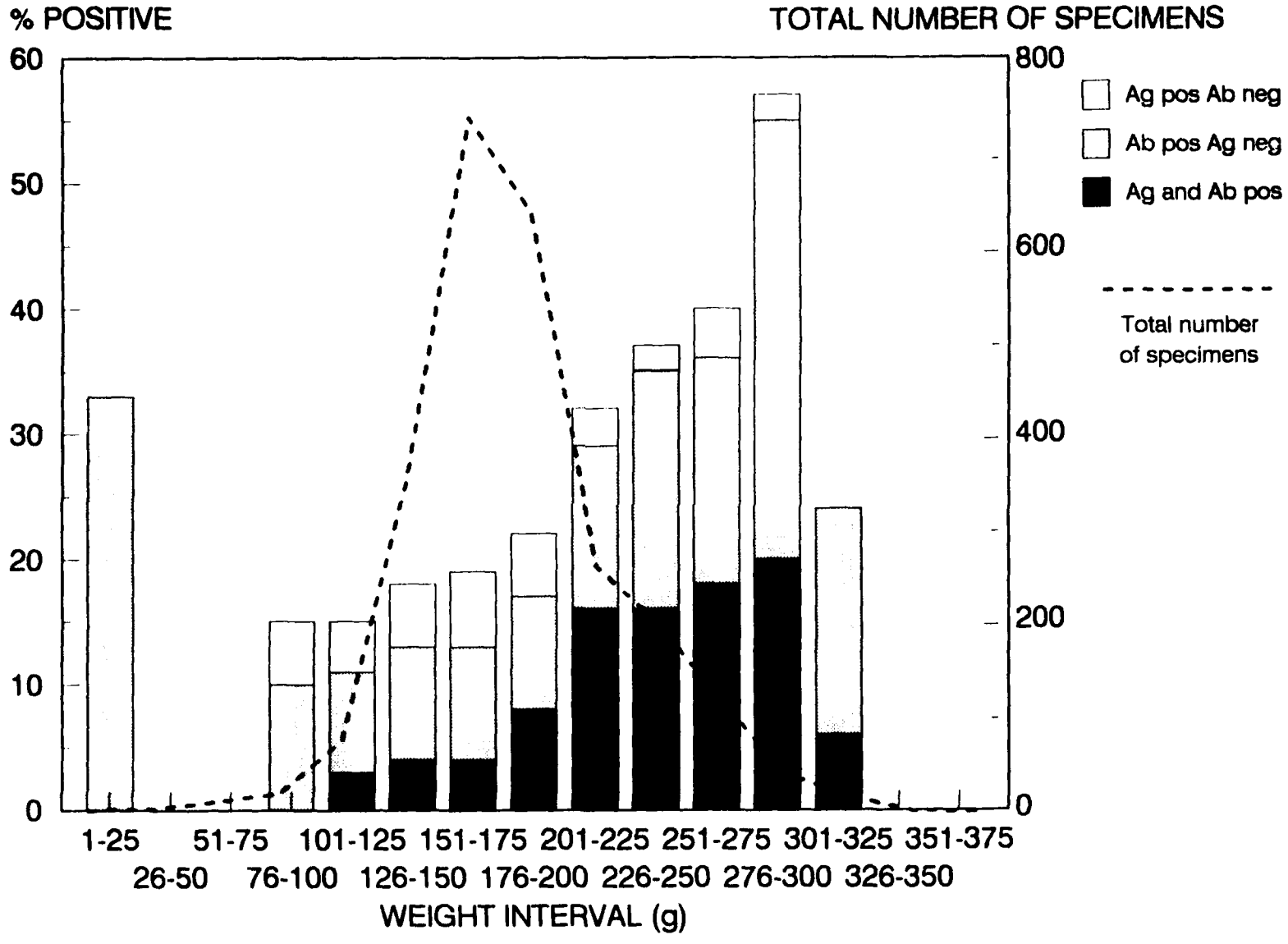
Figure 4 A. Areas were sorted based on bank vole abundance during the first cycle. The total number of bank voles trapped at the 16 locations were compared for cycle 1 and 2.

Figure 4 B. The infection rate cycle 1 and cycle 2 for areas sorted as in figure 4 A.

Figure 5. The number of C. rufocanus in different weight groups as well as the proportion of PUU virus infected individuals.

Figure 6. The number of and M. agrestis in different weight groups as well as the proportion of PUU virus infected individuals.

FIG 1


% POSITIVE
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60
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Total number of specimens
1-25
26-50
51-75
76-100
101-125
126-150
151-175
176-200
201-225
226-250
251-275
276-300
301-325
326-350
351-375
WEIGHT INTERVAL (g)

TAB 1

	Total	Ab pos (%)	Ag pos (%)	Ab or Ag pos (%)
CLET GLA	2493	478 (19%)	322 (13%)	599 (24%)
CLET RUF	620	58 (9%)	22 (4%)	77 (12%)
MICR AGR	367	18 (5%)	21 (6%)	37 (10%)
MYOP SCH	39	1 (3%)	4 (10%)	5 (13%)
APOD FLA	5	0	0	0
SOREX	377	NT	NT	NT

TOTAL 3901

Fig 2

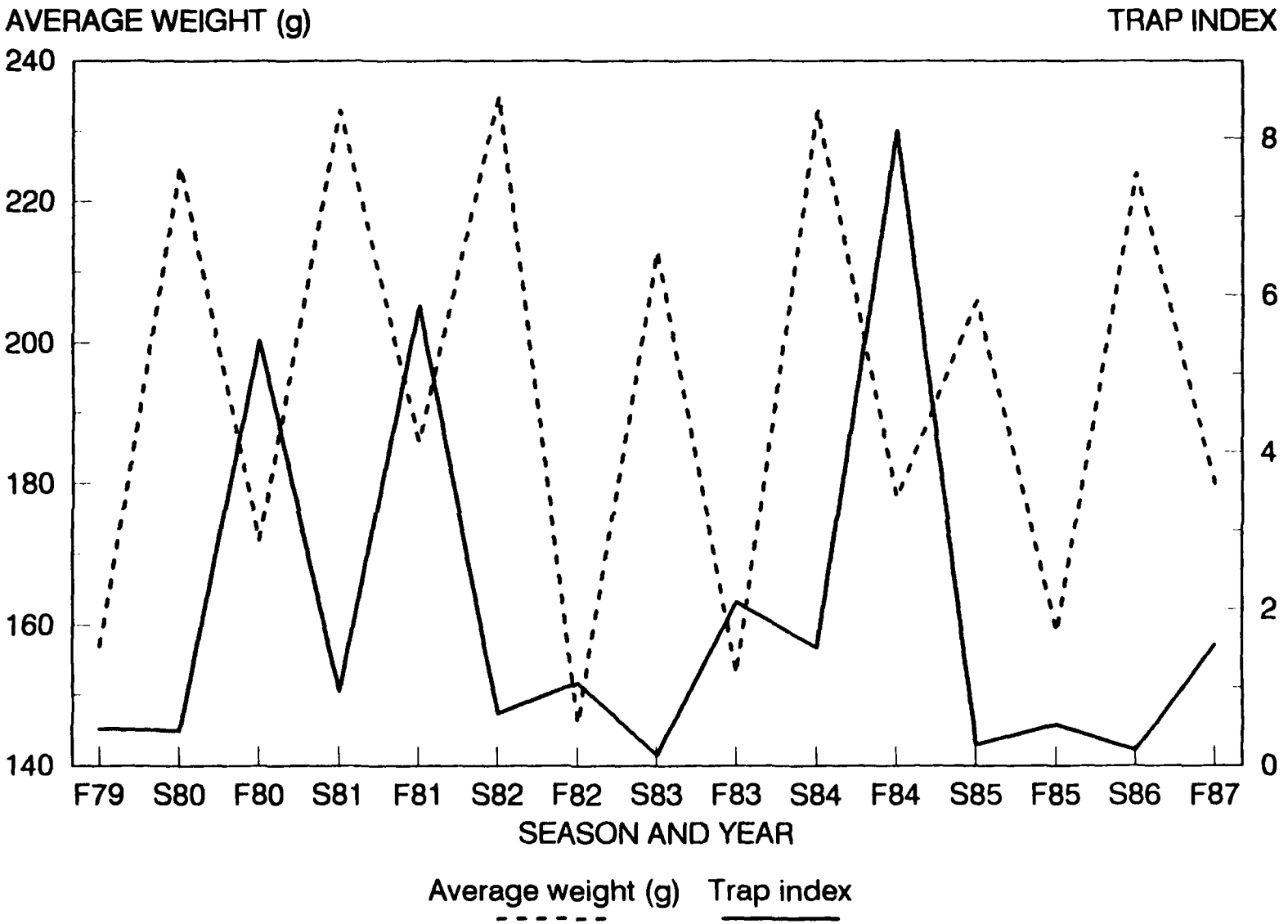

AVERAGE WEIGHT (g)
TRAP INDEX
240
220
200
180
160
140
8
6
4
2
0
F79 S80 F80 S81 F81 S82 F82 S83 F83 S84 F84 S85 F85 S86 F87
SEASON AND YEAR
Average weight (g)
Trap index

FIG 3

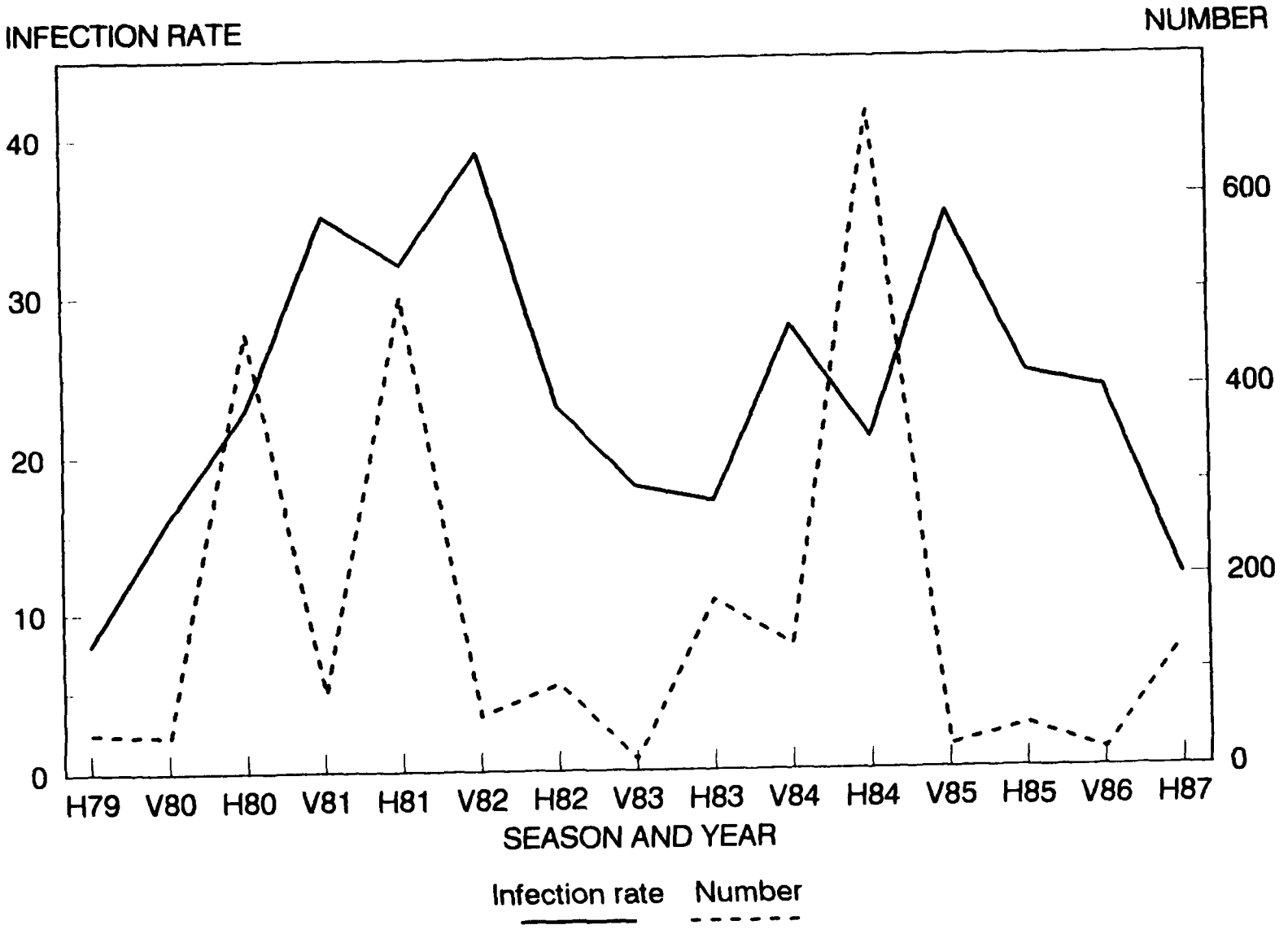

INFECTION RATE
NUMBER
40
30
20
10
0
600
400
200
0
H79 V80 H80 V81 H81 V82 H82 V83 H83 V84 H84 V85 H85 V86 H87
SEASON AND YEAR
Infection rate
Number

Fig 4

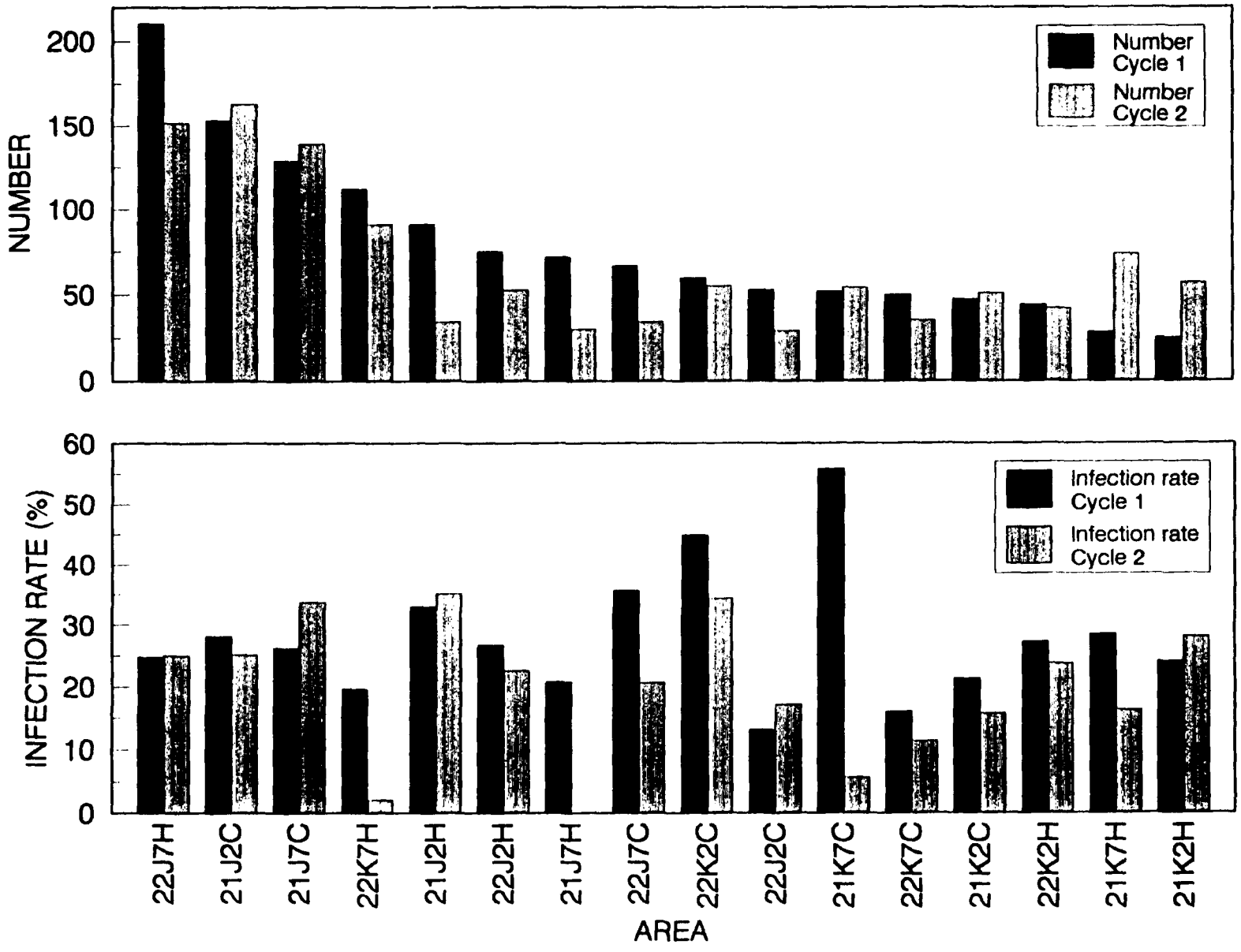

A
B
NUMBER
200
150
100
50
0
Number Cycle 1
Number Cycle 2
INFECTION RATE (%)
60
50
40
30
20
10
0
Infection rate Cycle 1
Infection rate Cycle 2
22J7H
21J2C
21J7C
22K7H
21J2H
22J2H
21J7H
22J7C
22K2C
22J2C
21K7C
22K7C
21K2C
22K2H
21K7H
21K2H
AREA

FIG 5

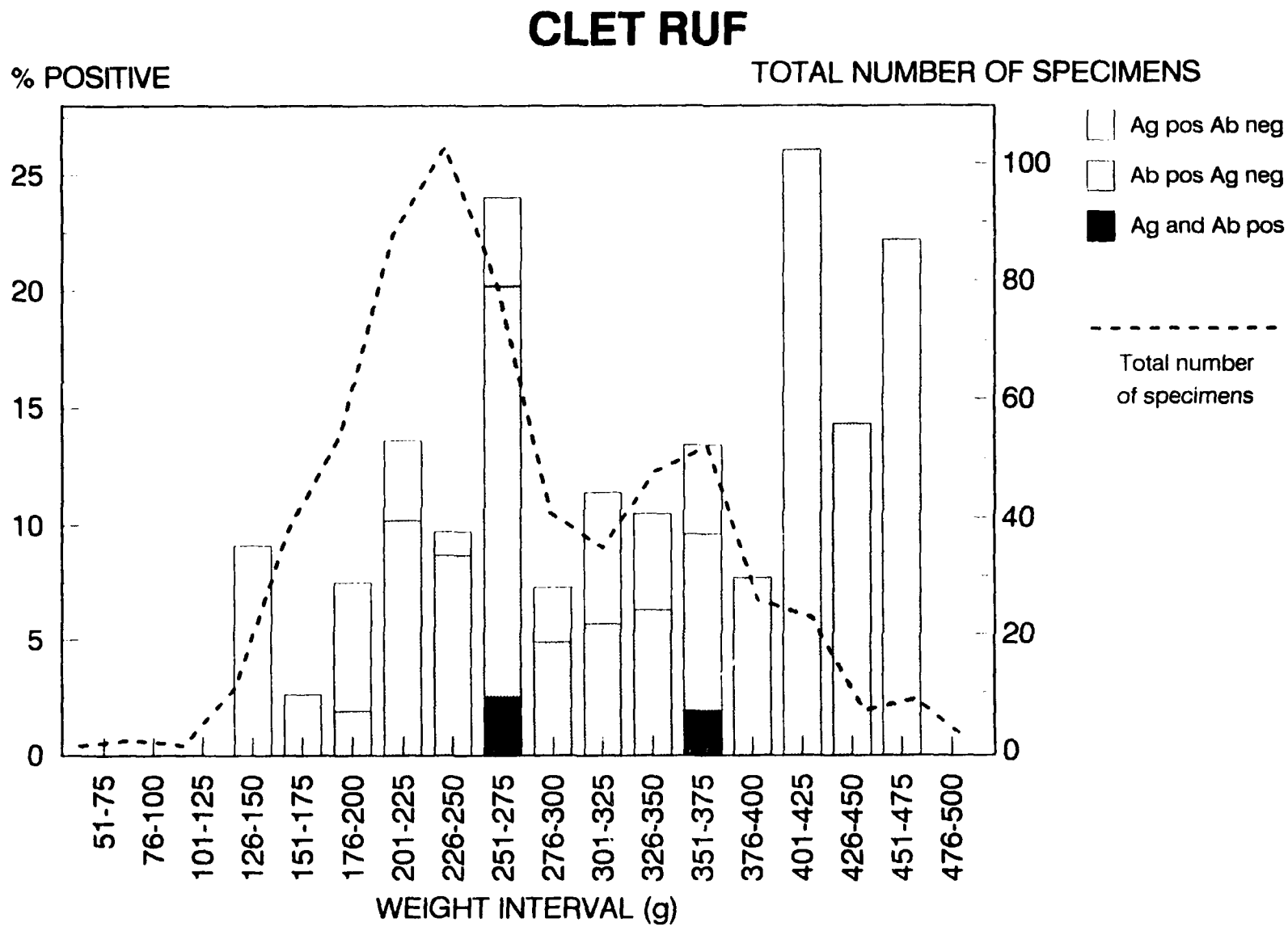
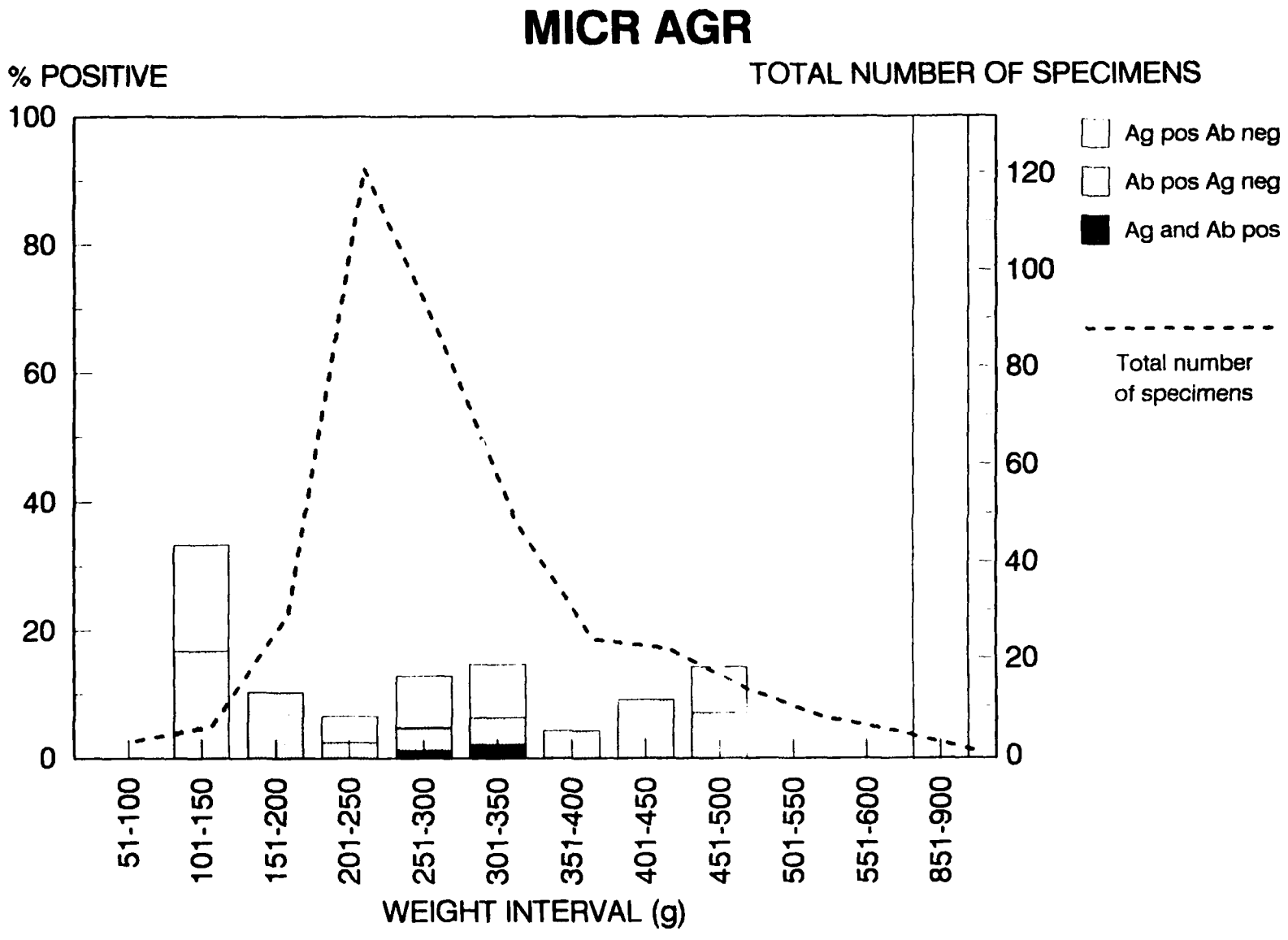

CLET RUF
% POSITIVE
TOTAL NUMBER OF SPECIMENS
25
20
15
10
5
0
100
80
60
40
20
0
Ag pos Ab neg
Ab pos Ag neg
Ag and Ab pos
Total number of specimens
51-75
76-100
101-125
126-150
151-175
176-200
201-225
226-250
251-275
276-300
301-325
326-350
351-375
376-400
401-425
426-450
451-475
476-500
WEIGHT INTERVAL (g)

FIG 6


MICR AGR
% POSITIVE
TOTAL NUMBER OF SPECIMENS
100
80
60
40
20
0
120
100
80
60
40
20
0
Ag pos Ab neg
Ab pos Ag neg
Ag and Ab pos
Total number of specimens
51-100
101-150
151-200
201-250
251-300
301-350
351-400
401-450
451-500
501-550
551-600
851-900
WEIGHT INTERVAL (g)