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## IMPACT OF VIRAL HEMORRHAGIC DISEASE ON A WILD POPULATION OF EUROPEAN RABBITS IN FRANCE

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**ABSTRACT:** An outbreak of rabbit viral hemorrhagic disease (RVHD) and of myxomatosis occurred in a free-living population of rabbits (*Oryctolagus cuniculus*) near Paris (France) in 1995. Annual mortality rates were 88% in adults and 99% in juveniles. There was no difference in mortality rates between males and females. Since most adults were protected with myxoma antibodies after May, they probably died of RVHD. Mortality lasted throughout the year despite high proportions of rabbits having developed myxomatosis and RVHD antibodies, which suggests that the combination of the two diseases and the immunosuppressive characteristics of myxoma virus could be responsible for the mortality caused by RVHD. The proportion of juveniles with RVHD antibodies increased with their weight. Seroconversion against RVHD occurred in spring and autumn.

**Key words:** Epidemiology, European rabbit, immunosuppression, myxomatosis, *Oryctolagus cuniculus*, rabbit viral hemorrhagic disease.

### INTRODUCTION

Rabbit viral hemorrhagic disease (RVHD) was first described in China in 1984 (Liu et al., 1984). It is caused by a calicivirus (Ohlinger and Thiel, 1991; Moussa et al., 1992) first reported in Europe in 1988. It is now endemic in large parts of the continent (Morisse et al., 1991; Mitro and Krauss, 1993), but its impact on wild rabbit populations remains poorly documented.

Research on RVHD mainly concerns the structure of the virus, its impact on domestic rabbits and improved vaccination. In wild populations of European rabbits (*Oryctolagus cuniculus*), most studies refer to the epidemiology and spread of the disease throughout Europe (Morisse et al., 1991; Villafuerte et al., 1995; Chasey and Trout, 1995). In France, RVHD first appeared in 1988 and is now endemic (Barrat et al., 1996; Artois et al., 1997). Only Villafuerte et al. (1994) studied mortality rates induced by an RVHD outbreak in Spain and measured the effect on the rabbit population.

An outbreak of RVHD occurred in 1995 near Paris (France) in a free-living European rabbit population monitored since 1989 for a long-term study. In the present study, we describe the mortality rates and the patterns of mortality, and we attempt to understand the development of immunity in this rabbit population.

### MATERIALS AND METHODS

The Chèvreloup arboretum (48°40'N, 01°60'E) is located in Ile de France (close to Paris, France) with an oceanic climate exposed to the continental influence. Mean annual rainfall is 606 mm and mean annual temperature is 10.3 C. It is a 200-ha park in which a 5-ha central study area is defined. A detailed description of the study area is given by Marchandeau et al. (1995).

Throughout 1995 rabbits were captured with traps. Fourteen trapping sessions were organized from January to September, about every 3 wk. In October one capture operation per warren was conducted using ferrets (*Mustela furo*). During each capture operation, every rabbit caught was sexed, weighed and a blood sample was taken on blotting-paper (Gilbert et al., 1989; Chantal and Gilbert, 1990). In addition, each animal was individually marked with

ear-tags the first time it was captured. The ear-tags [Tip-Tags® (Rockall-France, Vitré, France) for juveniles and Top-Tags® (Rockall-France) for adults] were covered with Scotchlite® (Rangheard, Vaulx-En-Velin, France) reflecting paper. Three methods of recapture included trapping, resighting at dusk near the warrens, and resighting at night with a spotlight. A combination of these methods allowed us to build the life history of each rabbit and then to monitor the changes in the population size (Wood, 1980; Cowan, 1987).

Blood was examined for myxoma and RVHD antibodies. The tests were performed using blood collected and dried onto blotting paper (Gilbert et al., 1989; Chantal and Gilbert, 1990). The paper was cut into discs and two of them were placed in each well of a flat-bottomed microtitre plate, to which 100 µl PBS had been added for serum extraction. This starting dilution was used in another 96-well microplate (Falcon Probind, Becton Dickinson, Meylan, France) for indirect enzyme-linked immunosorbent assay (indirect ELISA) testing. VP60 purified RVHD capsid protein produced in baculovirus/Sf9 cells, in a Pipe Buffer pH 6.4 (Laurent et al., 1994), and semi-purified myxoma virus (French hypervirulent T1 strain, Ecole Nationale Vétérinaire, Laboratoire de Pathologie Infectieuse, Toulouse, France) produced in RK13 cells and semi-purified on a 36% sucrose cushion in a TL20 buffer, were respectively used as antigens. Probind assay plates were coated for 16 hr with viral proteins (1 µg per well in 100 µl PBS pH 7.6) or only PBS (one column as "blank"). After three washings in PBS, free-binding sites were blocked by incubation in 25 mg/ml gelatina in PBS for 1 hr at 37 C. The plates were then washed three times in PBS-0.1% Tween 20, then two consecutive 1:2 dilutions of eluate in PBS-Tween were added (100 µl). Positive and negative serum standards were included on each plate. After 60 min incubation at 37 C and three washings in PBS-Tween, a 1:3,000 dilution in PBS-Tween of goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma Chemical, St Louis, Missouri, USA) was added (100 µl) for 1 hr at 37 C. Finally, after four washings in PBS-Tween, disodium p-nitrophenyl phosphate (Sigma Chemical) at a concentration of 1 mg/ml in 10% diethanolamine was used (100 µl) as substrate. After 12 min in the dark at room temperature, the enzymatic reaction was stopped by addition of 2N NaOH and optical densities were read with the help of a spectrophotometer (Dynatech, St Cloud, France) at a wavelength of 405 nm. The eluate sample titer was expressed as the inverse of the highest dilution for which the optical density

was greater than thrice the optical density of the negative serum standard. Titers  $\geq 50$  were considered as positive.

Dead rabbits were examined to determine the cause of their death (myxomatosis, RVHD, coccidiosis, etc.), at the Laboratoire Vétérinaire Départemental (Departmental Veterinary Laboratory) of Maine et Loire (Angers, France). Since the necropsy merely showed a pale liver and hemorrhagic lungs and trachea, the lesions might possibly be attributed to RVHD or to bacterial infection of the lungs. Therefore, the analyses were aimed at isolating pathogenic bacteria in the lungs or detecting RVHD by the HA test, RVHD virus hemagglutinating human erythrocytes (Pu et al., 1985). A 1:6 solution of liver tissue was homogenized in a pH 7 buffer (saccharose : 20 g; Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O : 2.58 g; KH<sub>2</sub>PO<sub>4</sub> : 0.52 g; H<sub>2</sub>O : qsp 1,000 ml) and centrifuged at 1,300 g for 15 min. Human type 'O' erythrocytes were washed 3 times in a 0.9% sodium chloride solution and centrifuged at 220–300 g for 10 min at 4 C after each washing. A suspension of 160 10<sup>6</sup> cells/ml was prepared in a 0.9% sodium chloride solution, corresponding to a 2% erythrocyte suspension. 25 µl of erythrocyte suspension were placed in each well of a microplate (Falcon Probind, Becton Dickinson, Meylan, France). Successive 1 : 2 dilutions of supernatant liver tissue were prepared in 0.9% sodium chloride solution, and 25 µl of each were added to each well of the microplate. In addition, three reference solutions were prepared with liver of RVHD infected rabbits, liver of RVHD non-infected rabbits, and human erythrocytes. The microplate was incubated 30 min at room temperature and the test was read after sedimentation of the reference erythrocyte solution.

Statistical analyses were computed with npSTAT (Praxème, St. Georges d'Orques, France) program. The monthly proportions of rabbits with RVHD or myxomatosis antibodies were compared using  $\chi^2$  or Fisher exact tests. The rabbits that had been captured in two consecutive months were excluded to obtain statistically independent samples.

## RESULTS

High rabbit mortality was recorded in 1995. From 205 adults alive on 1 January 1995, only 24 (12%) survived on 15 December 1995. Survival did not differ significantly ( $\chi^2 = 0.17$ , 1 df,  $P = 0.686$ ) between males (11%) and females (13%). Of 136 juveniles caught during the year, two (1%) survived on 15 December 1995. The difference between adult and juvenile sur-

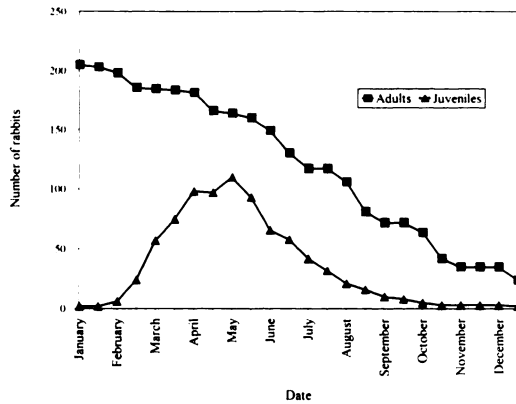


FIGURE 1. Temporal changes in size of the adult and juvenile populations of European rabbits (*Oryctolagus cuniculus*) during rabbit viral hemorrhagic disease and myxomatosis in France, 1995.

vival is statistically significant ( $\chi^2 = 12.17$ , 1 df,  $P \leq 0.001$ ). As for the adults, no difference was noted between the survival rate of males (2/75, 3%) and females (0/61, 0%) (Fisher exact test,  $P = 0.502$ ).

The pattern of mortality was quite different between the two age classes (Fig. 1). It was delayed and fast for juveniles (April to September) and more progressive and slower for adults (February to December). An outbreak of myxomatosis occurred in which 31 juveniles and three adults were caught with symptoms of myxomatosis from 24 May to 07 July.

Serological tests showed that a large proportion of the adult population had RVHD antibodies after March (Table 1): the proportion of adults with RVHD antibodies differed significantly between February and March ( $\chi^2 = 5.23$ , 1 df,  $P = 0.021$ ). The proportion of adults with myxoma antibodies did not differ significantly between February and March ( $\chi^2 = 0.04$ , 1 df,  $P = 0.831$ ), March and April ( $\chi^2 = 0.06$ , 1 df,  $P = 0.808$ ) and April and May ( $\chi^2 = 2.61$ , 1 df,  $P = 0.102$ ). In May and October respectively, 89 and 78% of the adults had RVHD antibodies and 89 and 85% had myxoma antibodies. Data were insufficient to obtain a reliable estimation of the proportion of rabbits with antibodies in June through September. Juveniles in April had myxoma antibodies (2/7, 29%). These observations suggests that both RVHD and myxomatosis were present after March and that myxomatosis became more virulent in May as indicated by the capture of rabbits with symptoms of myxomatosis.

The proportion of juveniles with antibodies (Fig. 2) increased by weight, and therefore with age (Marchandeaup et al., 1995). Only 23% ( $n = 36$ ) of rabbits weighing <400 g had RVHD antibodies, suggesting that most juveniles had no maternal antibodies or that these maternal

TABLE 1. Proportion of European rabbits with rabbit viral hemorrhagic disease (RVHD) and myxoma antibodies in France, 1995.

| Month              | Adults      |             | Juveniles   |             |
|--------------------|-------------|-------------|-------------|-------------|
|                    | RVHD        | Myxomatosis | RVHD        | Myxomatosis |
| January            | 0/2 (0%)    | 0/2 (0%)    |             |             |
| February           | 2/12 (17%)  | 6/12 (50%)  |             |             |
| March <sup>a</sup> | 16/26 (62%) | 14/26 (54%) | 0/3 (0%)    | 0/3 (0%)    |
| April              | 7/14 (50%)  | 8/14 (57%)  | 0/7 (0%)    | 2/7 (29%)   |
| May <sup>b</sup>   | 8/9 (89%)   | 8/9 (89%)   | 27/44 (61%) | 35/48 (73%) |
| June               | 1/1 (100%)  | 1/1 (100%)  | 14/20 (70%) | 15/20 (75%) |
| July               | 5/5 (100%)  | 5/5 (100%)  | 8/14 (57%)  | 13/14 (93%) |
| August             | 0/2 (0%)    | 2/2 (100%)  | 1/4 (25%)   | 4/4 (100%)  |
| September          | 2/4 (50%)   | 3/4 (75%)   | 4/6 (67%)   | 6/6 (100%)  |
| October            | 21/27 (78%) | 23/27 (85%) | 3/4 (75%)   | 3/4 (75%)   |

<sup>a</sup> Two adults which seroconverted both myxomatosis and RVHD were excluded for both myxomatosis and RVHD.

<sup>b</sup> Four juveniles which seroconverted RVHD were excluded for RVHD and one juvenile which seroconverted both myxomatosis and RVHD was excluded.

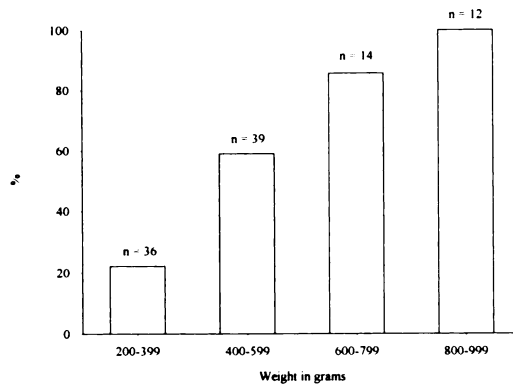


FIGURE 2. Proportion of young European rabbits (*Oryctolagus cuniculus*) with rabbit viral hemorrhagic disease antibodies by weight in France, 1995.

antibodies were undetectable. This proportion reached 100% ( $n = 12$ ) for juveniles weighing  $\geq 800$  g.

Several rabbits were caught repeatedly during the year and some of them seroconverted to RVHD. Therefore, it was possible to determine the period when these rabbits were exposed to the virus. Considering the smallest separate periods in which these seroconversions were detected, we obtained four short periods in which it was shown that the virus was present and active in the population. These seroconversions occurred in at least one rabbit between 1 March and 22 March, 13 April and 3 May, 4 May and 24 May and between 27 September and 18 October. Seroconversions were demonstrated for 20 of these rabbits, including 11 juveniles and 9 adults. Also, 36 juveniles seroconverted before their first capture and 23 seroconverted before 26 May. According to the low proportion of juveniles weighing  $< 400$  g with antibodies, it is probable that most of them actually seroconverted after being exposed to the virus. This confirmed the virus being present and active in spring. On the whole, 56 rabbits seroconverted but this value is underestimated because adults could have seroconverted before their first capture in 1995 and adults or juveniles could have seroconverted after their last capture.

Despite a careful and daily survey of the area, only one dead rabbit was found in the arboretum, close to the central study area (about 600 m), during the first fortnight of October; RVHD was confirmed as the cause of its death.

#### DISCUSSION

Two contagious diseases occurred on this territory in 1995—RVHD and myxomatosis. Particularly, an outbreak of myxomatosis began in March and increased from May to July. During this period 31 juveniles and three adults were caught with signs of myxomatosis. The decrease of the population, both in adults and juveniles, continued after this period. Moreover, from May 89% of adults and 73% of juveniles were protected by a previous contact as they had produced myxoma antibodies (Fenner and Woodroffe, 1953). These observations suggest that myxomatosis was not directly responsible for the overall mortality. However, we cannot exclude that it was partially responsible for the mortality in juveniles during the period of the outbreak since most of them were susceptible to this infection.

Timing of the RVHD seroconversions shows that the outbreak occurred in spring and autumn. Summer seroconversion also has been suspected, but could not be assessed due to sparse data during this period. We noticed that the duration of these seroconversions is consistent with the length of the period of population decrease. Furthermore, one dead rabbit was found and its analysis confirmed that RVHD was the cause of its death.

Since the adults were protected against myxomatosis, they likely died from RVHD. The proportion of juveniles with myxoma antibodies, 73% in May (Table 1), suggests that after May juveniles mainly died also from RVHD. Indeed, it would be an unsatisfactory explanation to assume that adults mostly died from RVHD and, during the same period, that juveniles mostly died from myxomatosis.

Despite most adults and juveniles hav-

ing RVHD antibodies from March and May, respectively, mortalities continued to occur throughout the year. We assume that the combination of the two diseases and the immunomodulating characteristics of myxoma virus may be related to the extent of the mortality, especially in adults. In fact, the immunosuppressive effect of the myxoma virus is now established and considered responsible for certain accidents occurring in some rabbit breeding centers using attenuated strains such as SG33 (Saurat et al., 1978) for vaccination (Brun et al., 1981). Recently, some viral structures were pointed out to be responsible for and accepted as a part of the viral pathogenicity (Mac Fadden et al., 1994; Turner et al., 1995; Petit, 1996). Therefore, we assume that simultaneous action of these two viruses increased the impact of RVHD on the population and explains the mortality till December (Fig. 1). The immunosuppressive effect of the myxomatosis was mentioned by Boag (1988) who suspected it being responsible for nematode and cestode infections.

The measured rate of mortality in adults during the outbreak of RVHD (88%), was among the highest recorded in the literature. In a free-living population monitored with a similar trapping protocol and affected by myxomatosis, the annual mortality rates were lower and differed between adult males (64%) and adult females (53%) (Cowan, 1987). In 1994, without RVHD, the mortality rate of the adults in our study area, calculated with the same method, was significantly lower (45%,  $\chi^2 = 89.96$ , 1 df,  $P < 0.001$ ; M. Guénézan and S. Marchandea, unpublished data), demonstrating the importance of the mortality caused by RVHD in 1995. However, when comparing 1994 and 1995, direct mortality by RVHD could be estimated at about 45%. In a wild population in Spain, Villafuerte et al. (1994) measured an average mortality rate of 55% in adults using radiotracking. In domestic rabbits, the mortality rate due to RVHD is generally 80 to 100% (Gregg et al., 1991; Cancellotti

and Renzi, 1991), but Loliger and Eskens (1991) recorded rates varying from 5% to >90% in Germany. Since we never previously observed any mortality due to RVHD in this area, it seems that this event is the first outbreak which occurred there and could explain the high mortality rate. Indeed, Villafuerte et al. (1995) noticed that mortality was high during the first epizootic (55 to 75%) and decreased (30%) 6 yr after the initial outbreak. We did not notice any difference in survival rates between males and females, or for adults and juveniles. These results agree with those recorded by Liu et al. (1984), Xu (1991) and Villafuerte et al. (1994), but are opposed to those of Rossell et al. (1989).

The pattern of mortality in adults differed from that previously described. The slow decrease of the adult population from April to December suggests that RVHD continued over 8 to 9 mo. This observation was confirmed by RVHD seroconversions which were noticed in March–May and in September–October. Sparse data prevented us from establishing if seroconversion occurred during the rest of the year. The observed duration of the outbreak is longer than any event previously published for wild populations. Villafuerte et al. (1994, 1995) recorded mortalities over 29 and 32 days and Rossell et al. (1989) recorded a duration of 42 days in domestic rabbits. However, Xu (1991) and Barrat et al. (1996) noticed that RVHD occurred throughout the year. Regarding such patterns of mortality, throughout the year, RVHD could be in some circumstances an endemic rather than an epidemic.

Both levels and patterns of mortality differed between adults and juveniles. The study of the birth date of the juveniles showed that the oldest juveniles were approximately 2-mo-old when mortality occurred in April, but we can not exclude that some rabbits of <2-mo-old could die from RVHD. However, this corroborates previous observations that the disease does not affect rabbits <1-mo-old and those 1- to 2-mo-old can be infected at a low per-

centage (Morisse et al., 1991; Xu et al., 1989; Xu, 1991). Alternatively, Villafuerte et al. (1994) did not observe any mortality due to RVHD in juveniles <4-mo-old. We noticed that 31 juveniles weighing <600 g, and approximately 2-mo-old (Marchandeau et al., 1995) produced RVHD antibodies. These rabbits were exposed to the virus but did not die from their first contact with it, suggesting that they were not susceptible. Their physiological status could not permit the development of the disease. It is now well established with regard to a first occurrence of RVHD that, whereas morbidity and mortality rates respectively approach and reach 100% and 90% in adults, juveniles younger than eight weeks survive infection. Some juveniles show high antibody titres and do not carry the virus (Gunn and Nowotny, 1996). Such juveniles might be suited to build up a new RVHD protected population but they are still susceptible to the myxoma virus. The beginning of the high mortality recorded in May could be related to the RVHD virus infecting juveniles which became physiologically susceptible. Later, during the course of June and July, the myxomatosis outbreak adds to RVHD as the cause of death: juveniles then died either from myxomatosis or from RVHD, possibly like in adults, after immunosuppression due to myxomatosis if they had RVHD antibodies.

Despite the overall high mortality rate, only one dead rabbit was found in the arboretum, although human surveys were thorough and the survey area was a park where the grass was regularly mowed which allowed easy detection of corpses. This suggests that most rabbits died in their warrens and that before death there was a change in behavior as noticed by Fuller et al. (1993) who observed that rabbits became lethargic before dying. Conversely, Villafuerte et al. (1994) collected about 300 dead rabbits at Doñana (Spain). We assess that in our case about 300 rabbits died in the central study area during this outbreak and it is unlikely that pred-

ators caught all these corpses before they were found by humans on this area. This difference in the expression of the disease could suggest the existence of different forms of RVHD with pathological changes in wild populations as is proposed in domestic rabbits (Xu et al., 1989; Cancellotti and Renzin, 1991; Fuller et al., 1993).

Presently we are attempting to study the changes in this rabbit population after the initial outbreak and to examine whether RVHD will become endemic and if the population will recover to its initial level.

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