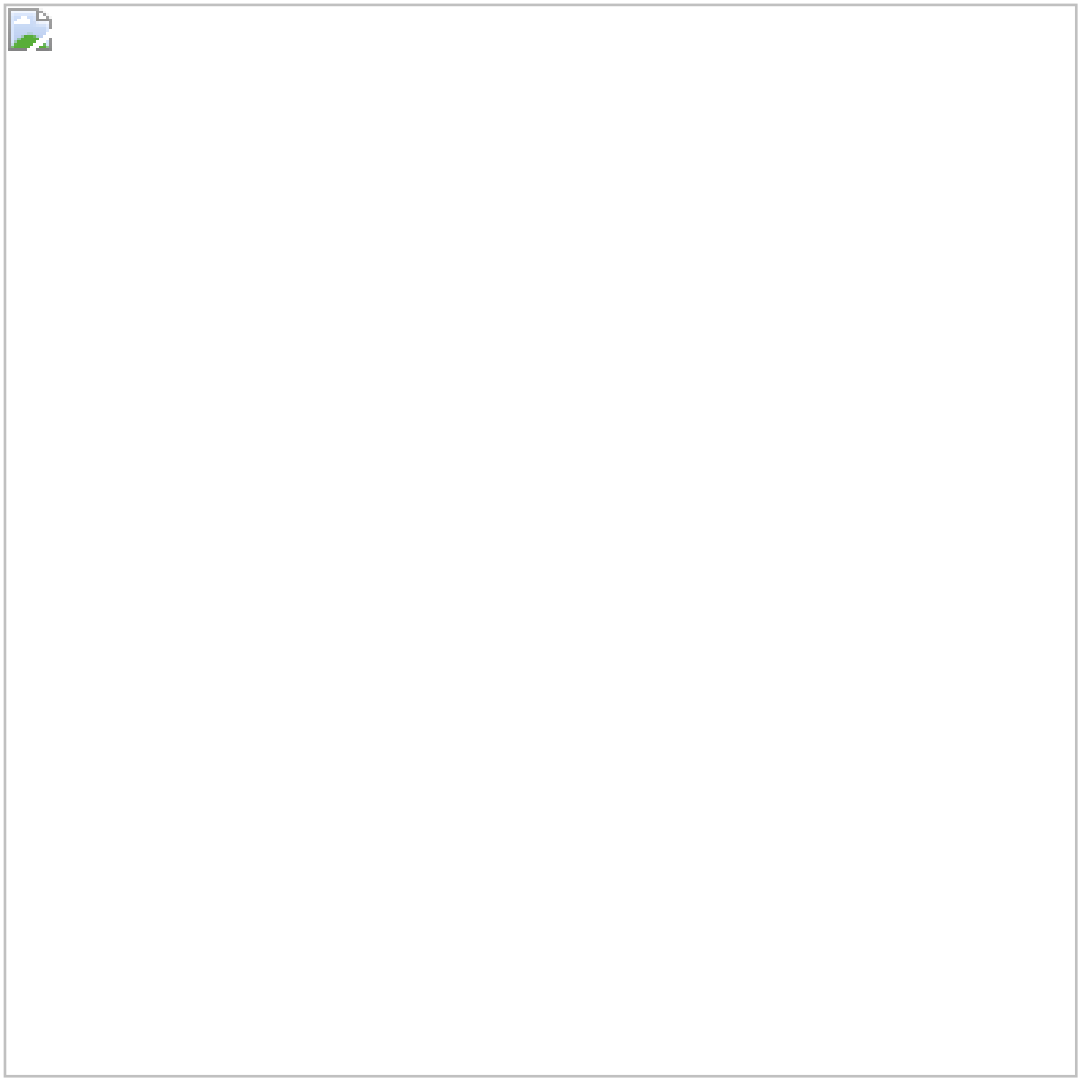

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CYTOPATHOLOGICAL OBSERVATIONS AND EPIZOOTIOLOGY OF FROG ERYTHROCYTIC VIRUS IN BULLFROGS (*RANA CATESBEIANA*)

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ABSTRACT: A 5 yr survey (1985 to 1989) revealed that the prevalence of frog erythrocytic virus (FEV) was significantly higher in juveniles than in adult bullfrogs (*Rana catesbeiana*) in Algonquin Park, Ontario (Canada). The prevalence of infection in juveniles declined during May and June and increased in early August, persisting until late September. Tadpoles were not infected naturally with FEV, but were susceptible to experimental infection. Experimental data indicate that FEV is neither waterborne, nor transmitted by the oral route, nor by the leech *Desserobdella picta*. The virus may be transmitted mechanically by the mosquito *Culex territans*, or the midge *Forcipomyia* (*Lasiohelea*) *fairfaxensis*. Nine percent recapture of marked uninfected frogs versus 4% recapture of infected animals suggests that FEV contributes to mortality of juvenile bullfrogs. Infected erythrocytes were transformed from ellipsoidal to spheroidal cells, some of which contained flattened elongate, trapezoidal inclusions. There was no evidence of denatured hemoglobin or significant change in the hemoglobin content of infected cells. Anemia recorded in heavily infected animals was not attributable to an increased osmotic fragility of infected erythrocytes.

Key words: Anemia, *Culex territans*, cytopathology, *Desserobdella picta*, *Forcipomyia fairfaxensis*, frog erythrocytic virus, prevalence.

INTRODUCTION

Although epizootiological and cytopathological information for erythrocytic viruses is scarce, some data are available for reptiles infected with species of *Pirhemocyton* (see Johnston, 1975) and *Toddia* (see Marquardt and Yaeger, 1967; Johnston, 1975), fish infected with piscine erythrocytic necrosis (PEN) virus (Appy et al., 1976) and epizootic haemopoetic necrosis virus (Langdon and Humphrey, 1987), and mammals infected with Colorado tick fever virus (Emmons et al., 1972; Bowen, 1988).

Studies on viruses infecting erythrocytes in amphibians were focused mainly on ultrastructure (Bernard et al., 1968; Desser and Barta, 1984; Gruia-Gray et al., 1989). Frog erythrocytic virus (FEV) occurs primarily in bullfrogs (*Rana catesbeiana*), although green frogs (*Rana clamitans*) and mink frogs (*Rana septentrionalis*) also are infected (Desser and Barta, 1984). As FEV is present in the peripheral circulation, a hematophagous vector is suggested. A leech (*Desserobdella picta*), a mosquito (*Culex territans*) and a biting midge (*Forcipomyia Lasiohelea fairfaxensis*), all of which feed on frogs in Algonquin Park, Ontario (Canada) are possible vectors of FEV.

The purpose of this study was to investigate the prevalence and mode of transmission of FEV. In addition, the morphological and physiochemical changes to FEV-infected erythrocytes were assessed.

MATERIALS AND METHODS

Epizootiology

From May to September of 1985 to 1989, 652 *Rana catesbeiana* adults, 2216 juveniles (<90 mm snout to vent length) and 200 tadpoles were caught in or near Lake Sasajewun (Algonquin Park, Ontario, Canada; 45°35′N, 78°30′W). Gender and snout to vent length were recorded for each frog. Frogs were tattooed with a letter-number system, allowing for their easy identification upon recapture.

The caudal vein in tadpoles or the maxillary vein of juvenile and adult frogs was pierced with a needle (26½g) and the blood that pooled at the pierced surface was collected in heparinized capillary tubes. Blood films were prepared by smearing the blood from the capillary tubes on a slide. The films were air dried, fixed in methanol for 5 to 10 sec, air dried and stained for 8

to 10 min with Giemsa stain (1:5 in phosphate buffer pH 7.2).

Experimental transmission

Giemsa-stained blood films of wild-caught tadpoles (n = 10), juvenile (n = 10) and adult (n = 10) frogs were made over a 3 wk period and examined for FEV inclusions. Two groups of five apparently uninfected individuals from each age class were inoculated intraperitoneally with 0.2 ml of either heparinized FEV-infected or uninfected (controls) blood. Blood films of experimental and control animals were examined for FEV inclusions at 2 day intervals over 3 wk.

To simulate the bite of an invertebrate vector, insect pins dipped in heparinized FEV-infected blood were used to prick the dorsal surface of four uninfected frogs (wild-caught juveniles monitored for 4 wk for the absence of FEV inclusions). Control experiments were conducted as above, using four uninfected wild-caught frogs and heparinized uninfected blood as the inoculum. Blood films from these animals were examined at 2 day intervals for 3 wk.

Mechanical transmission was tested using one infected and one uninfected frog tethered inside a cage covered with no-see-um netting. Ten to 20 wild-caught *F. fairfaxensis* were placed in the cage with the frogs, and four wild-caught *C. territans* were placed in a second cage with another two frogs. As controls, two uninfected wild-caught frogs were tethered in each of two cages containing either midges or mosquitoes as above. The frogs were removed from the cages only when the flies were observed to contain a blood meal. Frogs were exposed to the flies at midday, dusk and evening, to determine the peak feeding times. Blood films of the frogs were made at 2 day intervals over 3 wk.

As the midges often did not feed on the frogs in the cage, a wild-caught uninfected frog and an infected frog were tethered on a small board in a bog where midges and mosquitoes were observed to feed on frogs. The sentinel frogs were exposed to the midges and mosquitoes at midday, dusk and evening, to determine the peak feeding time. The same procedure was repeated five times with separate wild-caught and with lab-reared frog pairs.

Oral transmission was simulated by feeding two wild-caught uninfected frogs 65 μl infected heparinized blood or five midges which had fed on infected frogs. The midges were chilled briefly at 4 C to facilitate feeding them to the frogs. Blood from these animals was sampled at 3 day intervals over 3 wk, stained and observed as above.

To simulate waterborne transmission, two infected and two uninfected frogs were placed in each of five containers with sphagnum and a Petri dish of water for 3 mo. Blood from the uninfected frog was sampled at 1 wk intervals over a 3 mo period, stained and observed as above.

Electron microscopy of potential vectors

Several leeches (*Desserobdella picta*) which had fed on heavily infected frogs were fixed and prepared for electron microscopy according to the procedure of Desser and Weller (1977) at 1 hr, 12 hr and 1 day intervals up to 12 days post-feeding (PF). The salivary glands, intestine and crop were examined for FEV.

Culex territans larvae, collected from a pond near Lake Sasajewun, were reared to adulthood according to the procedure of Desser et al. (1973). Female mosquitoes and midges (*Forcipomyia fairfaxensis*) were primed for feeding (Desser et al., 1973) and placed in a small vial. The open end of the vial was placed on the dorsal surface of a heavily infected frog and the flies were removed once they had fed. On days 1, 2, 4, 5, 7, 9, 12, 14 and 22 PF, the salivary glands, midgut, and ovaries or eggs were embedded in 3% agarose, and fixed and prepared for electron microscopy as above.

Hematological tests

Blood was collected from the maxillary vein of each frog in heparinized capillary tubes, maintained at 4 C and examined within 5 hr. All hematological parameters were measured from each sample prior to determining the presence of FEV. Blood films were stained by the Giemsa method or prepared for Heinz body staining and examined by light microscopy. Fresh blood was examined by differential interference contrast (DIC) microscopy.

For hematological studies [erythrocyte count, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)], juvenile frogs were grouped into four categories according to the intensity of infection observed in Giemsa-stained blood films: uninfected frogs (n = 224) and frogs with less than 25% (n = 16), 50–75% (n = 20) and greater than 75% (n = 36) of erythrocytes infected with FEV.

Hematological tests (see Brown, 1976; Williams et al., 1983) included (1) verifying the presence of Heinz bodies (particles of denatured hemoglobin which attach to the erythrocyte cell membrane), (2) mean corpuscular hemoglobin (MCH, average weight of hemoglobin per red blood cell), (3) mean corpuscular hemoglobin concentration (MCHC, ratio of the amount of

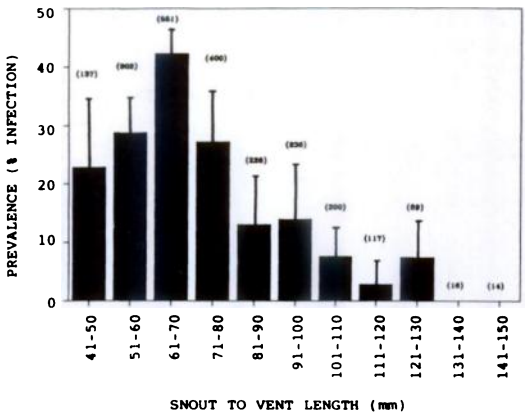


FIGURE 1. Prevalence of FEV in juvenile and adult *Rana catesbeiana*. Bars represent standard error.

hemoglobin to the red blood cell volume), (4) mean corpuscular volume (MCV, average volume per red blood cell denotes the MCV), (5) packed cell volume (PCV), (6) red blood cell count and (7) osmotic fragility.

To test for the presence of Heinz bodies, a drop of blood from each of 10 moderately infected frogs was placed on a coverslip and inverted on a slide containing a drop of crystal violet. The samples were examined after 5 min with a 40× objective of a light microscope.

The MCH was determined by making a 1:250 dilution of heparinized infected and uninfected blood in modified Drabkin's Reagent (Uno-Heme® kits, Becton Dickinson and Co., Rutherford, New Jersey 07070, USA). The absorbance of the hemoglobin was measured at 540 nm and compared to a calibration curve. The red blood cell count was determined by hemocytometry.

To evaluate the MCHC, the hemoglobin content was determined as above. The packed red blood cell volume (PCV) was determined by spinning the blood in a hematocrit centrifuge for 5 min at 1,000 g. The ratio of the volume of erythrocytes to the total volume (erythrocytes, buffy layer and serum) was measured.

The MCV was determined by taking a ratio of the PCV to the red blood cell count.

For the osmotic fragility test, juvenile frogs were categorized as uninfected (n = 197), low (n = 29), medium (n = 40) and high (n = 64) infections. Heparinized capillary tubes were used to collect 130 ± 10 μl of blood. Using the procedure described by Maley and Desser (1977), the blood cells were washed three times in 5 ml of isotonic (0.6%) phosphate buffer saline (PBS). The cells were resuspended in 1.5 ml of isotonic PBS. Aliquots of 200 μl of cells were added to dilutions of 1% PBS (water, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8%) to total volume of 3 ml. The samples were incubated at 41 C for 1 hr and overnight at 4 C. The resuspended cells were centrifuged at 1,000 g for 10 min and the absorbance of the supernatant measured at 540 nm.

A 2-sample, between group analysis of variance was used to test the statistical significance of these data. The data were analyzed at 95% confidence level.

For convenience, the surface area of the erythrocytes was calculated as length by width. Twenty infected and uninfected erythrocytes were measured for each of 16 Giemsa-stained blood films. The data on the red blood cell size were analyzed using Student's t-test.

RESULTS

Epizootiology

The prevalence of FEV infections increased from 0% in tadpoles (not shown on graph) to 29% in juveniles measuring 51–60 mm, and reaching its highest peak (42%) in juvenile frogs 61 to 70 mm. A lower prevalence of FEV occurred in juvenile frogs measuring 71 to 90 mm. Lower still was the prevalence in adults (91–130 mm), with no infection observed in adult frogs 131–150 mm (Fig. 1). Of the total juvenile population sampled 30% were infected, whereas 9% of the total adult population sampled were infected. Over the 5 yr study period, 9% of the uninfected juvenile bullfrogs and 4% of the infected juvenile bullfrogs were recaptured. There was no correlation between prevalence and gender.

The seasonal prevalence of FEV infections was determined only in juveniles because of the above mentioned higher prevalence of infection. A prevalence of 28% was observed in late May/early June, and dropped as the temperature rose through June and July, remaining at approximately 13% until the end of July. A rise in the prevalence occurred at the beginning of August, reaching 62% by the end of the sampling period in September (Fig. 2).

Transmission studies

Although tadpoles were not naturally infected with FEV, infections were in-

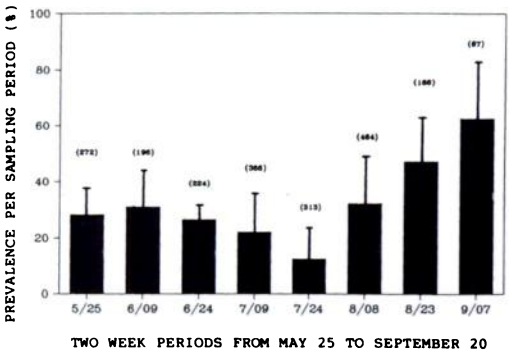


FIGURE 2. Seasonal prevalence of FEV in juvenile *Rana catesbeiana* over 2 wk periods during 1985 and 1989. Bars represent standard error.

duced by intraperitoneal inoculation of heavily infected blood. Within 14 to 21 days post-inoculation, the blood of the five tadpoles in the experimental group contained FEV inclusions. None of the tadpoles in the control group became infected (Table 1).

Two of the five juveniles in the experimental group became infected within 7 to 10 days post-inoculation. All the juveniles in the control group remained uninfected. Inoculation of blood into adult frogs in both the experimental and control groups did not result in viremia (Table 1).

Attempts to simulate mechanical transmission of FEV (by pin prick) to uninfected frogs resulted in all the frogs becoming infected within 7 days post-inoculation. Control animals remained uninfected (Table 1).

The peak feeding interval in the bog for both *C. territans* and *F. fairfaxensis* occurred between mid-afternoon and dusk. The uninfected frog, exposed to the mosquitoes, did not become infected, whereas, the uninfected frog exposed to the biting midges became infected. Viral inclusions were, however, observed in both midge and mosquito control frogs (Table 1).

Within 10 days post exposure to the bites of midges, two of five wild-caught frogs, tethered in the bog, became infected with FEV. None of the five lab-reared frogs exposed to midges became infected (Table 1).

TABLE 1. Summary of FEV transmission studies.

Treatments	FEV transmission	Number of experiments
Intraperitoneal inoculation of FEV		
in 5 tadpoles (wild-caught)	+ (100%)	1
in 5 juveniles (wild-caught)	+ (40%)	1
in 5 adults (wild-caught)	–	1
controls (15 wild-caught)	–	1
Mechanical Transmission		
4 wild-caught juveniles pricked with insect pin dipped in infected blood	+ (100%)	1
4 wild-caught controls pricked with insect pin dipped in uninfected blood	–	1
1 infected and 1 wild-caught uninfected frog in cage with *Culex territans*	–	5
1 infected and 1 wild-caught uninfected frog in cage with *Forcipomyia fairfaxensis*	+ (100%)	5
control: 2 uninfected wild-caught frogs in cage with *C. territans*	+ (100%)	5
control: 2 uninfected wild-caught frogs in cage with *F. fairfaxensis*	+ (100%)	5
1 infected and 1 wild-caught uninfected frog in bog with *F. fairfaxensis*	+ (40%)	5
1 infected and 1 lab-reared uninfected frog in bog with *F. fairfaxensis*	–	5
Oral Transmission		
2 wild-caught frogs ingesting infected blood	–	1
2 wild-caught frogs ingesting *F. fairfaxensis* which fed on infected frogs	–	1
controls: 4 wild-caught frogs used in both oral transmission experiments inoculated with infected blood	+ (100%)	1
Waterborne Transmission		
2 infected and 2 uninfected wild-caught frogs housed in same container	–	5
controls: 10 wild-caught frogs used in waterborne transmission experiments inoculated with infected blood	+ (50%)	1

TABLE 2. Erythrocyte parameters for uninfected and FEV-infected bullfrogs.

Parameter	Uninfected[a]	Infected[a]
Size (μm)	23.1 (0.9) × 15.3 (0.7) (n = 320)	19.5 (1.1) × 13.7 (0.8) (n = 320)
Surface Area (μm²)	353.5 (28.7) (n = 320)	266.5 (27.6) (n = 320)

Parameter	Uninfected[a] n = 224	FEV +[a,b] n = 16	FEV ++[a,c] n = 20	FEV +++[a,d] n = 36
RBC count[e] (×10⁶/mm³)	0.4 (0.2)	0.3 (0.1)	0.3 (0.1)	0.2 (0.1)
PCV[f] (%)	23.6 (0.1)	22.9 (0.1)	24.2 (0.1)	22.1 (0.1)
MCV[g] (μm³)	754.4 (261.5)	736.1 (227.0)	933.3 (221.6)	938.9 (380.2)
MCH[h] (pg)	197.0 (65.4)	197.5 (80.0)	204.4 (65.1)	224.3 (68.6)
MCHC[i] (%)	27.1 (11.1)	26.6 (5.1)	23.3 (7.7)	24.7 (4.9)

[a] Mean (standard error); [b] <25% FEV infected cells; [c] 50%–75% FEV infected cells; [d] >75% FEV infected cells; [e] Red blood cell count; [f] Packed cell volume; [g] Mean corpuscular volume; [h] Mean corpuscular hemoglobin; [i] Mean corpuscular hemoglobin content.

Oral and waterborne transmission experiments did not result in infections. All the animals in the oral transmission experiment became infected following intraperitoneal inoculation of FEV-infected blood. Of the 10 animals used in the waterborne experiment, five became infected following intraperitoneal inoculation with infected blood (Table 1).

Electron microscopy of potential vectors

Virus particles were observed in the blood meal of *D. picta*, up to 12 hr PF. Neither virus particles nor assembly sites, however, were observed in the tissues of the leech, mosquito or midge.

Morphological changes to erythrocytes

Uninfected erythrocytes were ellipsoid, containing a homogeneous cytoplasm and central nucleus (Fig. 3). Most Giemsa-stained, FEV-infected erythrocytes were more spheroidal. A dense, often displaced nucleus, acidophilic cytoplasmic inclusions, and an opaque vacuole characterized these cells (Fig. 4). FEV inclusions, as well as occasional large, flattened, elongate structures, which spanned the long axis of the erythrocyte were observed using DIC optics (Fig. 5). In most infected frogs less than 50% of the cells harbored viruses; up to 90% of the erythrocytes were infected in some animals.

Heinz body stain, MCH and MCHC

There was no evidence of denatured hemoglobin as indicated by the absence of Heinz bodies. However, a large, trapezoidal inclusion, spanning the length of some erythrocytes was highlighted with the Heinz body stain (Fig. 6). Occasionally, more than one inclusion per cell was observed at the electron microscopic level (Gruia-Gray et al., 1989).

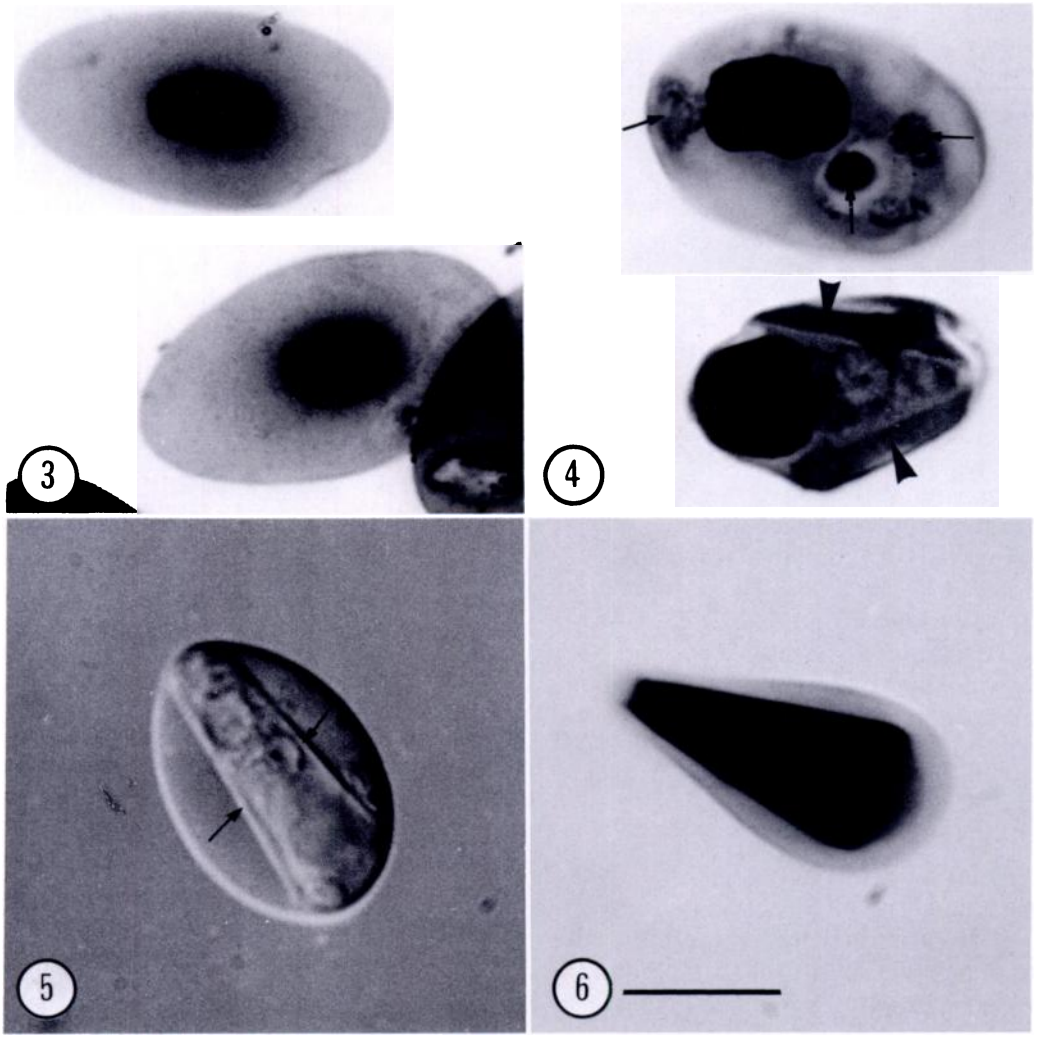
No significant difference was apparent among the MCH values of uninfected frogs and frogs with different degrees of FEV infections (Table 2). Likewise, there was no significant difference among the MCHC values of uninfected and infected animals (Table 2).

Red blood cell count

A significant difference (95% confidence level) in the red blood cell count was observed between uninfected frogs and all the infected frogs sampled. The red blood cell count in frogs with the highest intensity of infection was approximately 28% lower than that of uninfected frogs or frogs with a lower intensity of infection (Table 2). At times, approximately 30% more immature erythrocytes were observed, some of which were infected with FEV.

Red blood cell size, packed cell and mean corpuscular volumes

In addition to a spheroid shape, other changes were recorded in infected eryth-



FIGURES 3–6. Photomicrographs of uninfected and FEV-infected bullfrog erythrocytes. Bar = 10 μm for all figures. 3. Giemsa's-stained uninfected erythrocytes with homogeneous cytoplasm and central nucleus. 4. Giemsa's-stained FEV-infected erythrocytes with numerous cytoplasmic inclusions (arrows) and a displaced dense nucleus. The lower erythrocyte contains a large flattened, quadrilateral inclusions (large arrow heads). 5. Differential interference contrast micrograph showing a variety of cytoplasmic inclusions. Note the large flattened body (arrows), which spans the length of the cell. 6. Heinz body-stained erythrocyte showing a flattened inclusion which spans the cell.

rocytes (Table 2). The surface area of infected cells averaged 25% lower than uninfected ones (Table 2). No significant differences were observed in the PCV of uninfected versus infected frogs. Frogs with a moderate or high intensity of infection demonstrated a 20% increase in MCV. The cytoplasm of FEV-infected erythrocytes stained more intensely than that of uninfected ones.

Osmotic fragility

No significant difference was observed between the osmotic fragility of uninfected and infected erythrocytes.

DISCUSSION

The greater frequency and intensity of FEV infections in juvenile bullfrogs, coupled with the fact that adults were refrac-

tory to experimental infections, suggest that older frogs acquire immunity following repeated exposure to the virus. The incubation period of FEV in frogs increases with size in a similar fashion to PEN virus in fish (MacMillan and Mulcahy, 1979). Fish inoculated intraperitoneally with PEN virus became infected 2 to 25 days postinoculation (Evelyn and Traxler, 1978). The prevalence of FEV in frogs was similar to that reported for PEN virus in fish of different age classes. Smail (1982) suggested that the lower prevalence of PEN virus infections in older fish and apparent recovery from the infection by some fish may, in part, be due to an immune response.

The marked increase in infections of recently metamorphosed juveniles in mid to late July suggests that FEV is transmitted in mid summer, a period when the leech, mosquito and midge, are feeding on frogs. While *D. picta* commonly feeds on bullfrog tadpoles, naturally infected tadpoles were not seen (despite their susceptibility to experimental infection), suggesting that the leech does not act as a vector for the virus. Although evidence for the mode of transmission of FEV is equivocal, the data indicate that the virus is neither waterborne nor orally transmitted. Failure to detect viral replication in the tissues of the flies along with the limited data from transmission experiments, suggest that the mosquito or the midge may transmit the virus mechanically.

The cytoplasm of many infected erythrocytes contained, in addition to the viroplasm and viral particles (Desser and Barta, 1984; Gruia-Gray et al., 1989), a prominent inclusion resembling crystalline hemoglobin observed in the cod erythrocytes (Thomas, 1971). Alternately, these inclusions, as shown by DIC microscopy and crystal violet staining to be flattened and elongate, may be aggregates of residual viral proteins, as the inclusions associated with FEV were similar to those described from cells infected with other viruses (Sousa and Weigl, 1976). The prominent flattened inclusions, virus particles and viroplasm may account for the observed increase in the MCV of infected cells.

In blood films, infected erythrocytes appeared smaller and more spheroidal than uninfected cells. The insignificant differences in the PCV between uninfected and infected frogs and the two-dimensional measurement of the infected cells may, however, be misleading. As MCV represents a more accurate measurement of volume than PCV, the increase in MCV and in the intensity of staining of infected erythrocytes suggest that these cells have a greater volume. Thus, from a three-dimensional perspective, infected cells are larger than uninfected ones. The transformation of infected erythrocytes from flattened, ellipsoidal to spheroidal was also recorded for erythrocytes of fish infected with the PEN virus (Appy et al., 1976). These structural alterations may be due to disruption of the erythrocyte cytoskeleton. It has been shown that several viruses, including frog virus 3 (Murti and Goorha, 1983), African swine fever virus (Carvalho et al., 1988) and adenovirus (Luftig, 1982) alter the cytoskeleton of their host cells.

Anemia, characterized by a significant reduction in the erythrocyte counts in heavily infected frogs was correlated with FEV infections. The observed anemia was apparently not the result of increased osmotic fragility of infected erythrocytes and may be due to phagocytosis of distorted, infected cells. This was, however, not determined in this study. Although the hemoglobin concentration (MCH and MCHC) appeared unchanged in infected cells, their oxygen carrying capacity might have been altered.

The cytopathology and anemia recorded in FEV-infected bullfrogs along with the lower recapture rate of marked infected animals, indicate that FEV may contribute to mortality of juveniles. Heavily infected frogs may be more susceptible to predation and infections with other potential pathogens.

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