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## POXVIRUS INFECTION OF THE MANX SHEARWATER (*PUFFINUS PUFFINUS*)

Patricia A. Nuttall,<sup>1</sup> M. De L. Brooke,<sup>2</sup> and C. M. Perrins<sup>2</sup>

**ABSTRACT:** During studies on the etiology of puffinosis, a disease of the Manx shearwater, 1 to 4% of full-grown birds were found to have dry, non-pigmented lesions on the webs of the feet. Poxvirus infection was detected in six of seven full-grown birds with such lesions. The lesions contained large encapsulated inclusions which were packed with mature and immature poxvirus particles. Poxvirus infection was not apparent in shearwater fledglings during puffinosis epizootics, and its spatial distribution was not related to that of puffinosis. The results indicate that poxvirus infection produces a mild, self-limiting disease in shearwaters and is not the cause of puffinosis.

### INTRODUCTION

Skomer Island, situated off the southwestern coast of Wales, supports approximately 95,000 breeding pairs of Manx shearwaters (*Puffinus p. puffinus*) (Alexander and Perrins, 1980). Each year, during the period from late August until mid-September, epizootics of the disease known as puffinosis affect young shearwaters (Dane, 1948; Nuttall et al., 1982). The prevalence of the disease varies markedly with geographical location on the island, occurring primarily in areas of thick vegetation (Nuttall et al., 1982). Many of the affected birds have blisters on the webs of the feet (Miles and Stoker, 1948); paralysis and locking of the ankle joint of the leg ("extensor spasm"), and conjunctivitis, have been described also (Dane, 1948; Dane et al., 1953). Previous attempts to determine the etiology of puffinosis resulted in the isolation of a virus from blood and blister fluid of diseased shearwaters, and from mites (*Neotrombicula autumnalis* Shaw) removed from diseased birds (Miles and Stoker, 1948; Stoker and Miles, 1953). During studies on puffinosis, we found evidence of poxvirus infection. The

relationships between this virus and puffinosis are discussed in the present paper.

### MATERIALS AND METHODS

#### Shearwaters

On Skomer Island, birds were captured on the ground at night and examined by torchlight. Some birds were found to have thickened area(s) on the web, but otherwise appeared healthy. These birds were released after samples were taken, using a scalpel, from very prominent areas of thickening, care being taken not to damage the web. The proportion of birds with thickened lesions of the webs was assessed by catching several hundred shearwaters in June 1982, March 1983, and in June/July 1984 in areas where puffinosis is prevalent (North Valley), and where it is absent (Neck; Nuttall et al., 1982). The mid-summer samples contained large numbers of immature birds (2 to 5 yr old) whereas the March sample included birds of breeding age, at least 5 yr old (Perrins et al., 1973). Birds that showed clinical signs of puffinosis (blisters on the webs and/or extensor spasm) were retained and transported to Oxford for autopsy. In August 1979, six shearwater nestlings approximately 65 days old were collected from Skomer under license from the Nature Conservancy Council and transported to Oxford. They were kept under conditions approved by the Home Office.

#### Cell cultures

Primary cultures of chick embryo liver and kidney cells were derived from 14-day-old fertile hens' eggs, and chick embryo fibroblasts from 8-day-old eggs. They were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Explant cultures were established from shearwater organs and webs by placing 1-mm<sup>3</sup> tissue samples in 25-cm<sup>2</sup> flasks, leaving the

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flasks at room temperature for 1 hr to allow the tissue samples to adhere, and then gently adding to each flask 5 ml EMEM supplemented with 20% heat-inactivated FCS. The flasks were kept at 37 C in a CO<sub>2</sub> incubator, and the medium changed weekly. When confluent monolayers were formed, the cells were subcultured and the FCS concentration reduced to 10%.

#### Inoculation of cell cultures and eggs

The skin between digits of the web was removed after necropsy, cut into small pieces, and then homogenized in phosphate buffered saline containing 0.4% bovine plasma albumin, 200 units/ml penicillin and mycostatin, 200 µg/ml streptomycin and 200 µg/ml kanamycin. After clarification, 0.2 ml of each homogenate were inoculated onto the chorio-allantoic membrane (CAM) of either embryonated hens' eggs (11 days old unless otherwise specified), shearwater eggs (four eggs approximately 12 days old, four eggs 18 days old, and five eggs 25 to 37 days old, collected under licence from the Nature Conservancy Council), or six herring gull (*Larus argentatus*) eggs (approximately 13 days old). Three days after inoculation the CAM's were harvested and examined microscopically.

#### Electron microscopy

Samples (approximately 1 mm<sup>3</sup>) of webs (from either blistered or thickened areas) and inoculated CAM, and pelleted inoculated cell cultures, were fixed in 2% glutaraldehyde followed by 1% osmic acid and then dehydrated in a graded series of ethanol followed by acetone, and embedded in Emix (C210 resin and C211 hardener from Emscope Laboratories Ltd., Kingsnorth Industrial Estate, Wotton Road, Ashford, Kent TN23 2LN, United Kingdom). Sections were stained with lead citrate and uranyl acetate and examined using a JEOL 100CX electron microscope at 100 kV.

### RESULTS

#### Examination of shearwaters

Poxvirus was detected by electron microscopy in webs from six birds examined (Table 1). All the birds were full-grown and only one (designated SH79-1) exhibited clinical signs characteristic of puffinosis. SH79-1 was a 4-yr-old female (ringed as a fledgling on 26th August 1975 on Skokholm Island, Wales) and was found on the night of 22nd April 1979. Dried remains of blisters were observed on the



FIGURE 1. Ventral surface of web of a Manx shearwater showing lesion caused by poxvirus.

webs and tarsi and the bird showed signs of respiratory distress. No obvious lesions were observed on necropsy of SH79-1 and of diseased fledglings. The remaining poxvirus-infected birds did not show signs of puffinosis and appeared healthy. They had hard, dry, non-pigmented lesions which stood proud of the surface of the web (dorsal and/or ventral surface) and were 1 to 10 mm in diameter and up to 3 mm thick (Fig. 1).

Of the birds examined in June 1982, 3.88% (17/438) bore lesions but the prevalence was not significantly different in areas of Skomer where puffinosis is observed (North Valley: 11/296 or 3.72%) and where puffinosis is absent (Neck: 6/146 or 4.11%). A similar prevalence (15/411 or 3.65%) was observed in June/July 1984. Both these mid-summer samples included a significantly higher proportion ( $\chi^2$  tests,  $P < 0.01$  in both cases) of birds having lesions than did the sample caught in March 1983. Then 5/492 or 1.02% of birds examined bore lesions.

#### Shearwater explant cell cultures

Cell cultures were established from the webs of a total of 12 shearwater fledglings showing signs of puffinosis and collected in 1979, 1981, 1982 and 1983. Once established, the cultures were passaged several times (SH79-1 web explant culture

TABLE 1. Shearwaters examined by electron microscopy for the presence of poxvirus infection of the web.

Dates of collection	Approximate age <sup>a</sup>	No. birds examined	No. with poxvirus <sup>b</sup>
2 Sept. 76	70 days	1	0
10 Sept. 77	70 days	1	0
24 Apr. 79	≥2 yr	1	1
14–15 Sept. 79	70 days	8	0
12 June 82	≥2 yr	1	1
13–14 Sept. 82	70 days	7	0
16–19 Mar. 83	≥5 yr	5	4
7 Sept. 83	70 days	8	0

<sup>a</sup> Birds examined during September were fledglings showing clinical signs of puffinosis. Those examined earlier in the year were full-grown breeding and non-breeding birds with lesions.

<sup>b</sup> Poxvirus infection detected in web samples by electron microscopy.

was passaged 17 times). Poxvirus was not observed in any of the cultures examined at primary and subsequent passage levels by electron microscopy.

#### Inoculation of cell cultures and eggs

Extracts of webs from birds collected in 1977, 1978 and 1979 (Table 1) were inoculated onto CAM of embryonated hens' eggs. Small (approximately 0.5 mm in diameter) white opaque pock-like lesions were observed on the CAM 3 days after inoculation of two 18-day-old eggs with homogenized web of SH79-1; the embryos were alive. Web extracts from the remaining birds produced various effects when inoculated onto CAM, including thickening of the membrane, hemorrhage, and sometimes indistinct "spots," with or without death of the embryo; none produced the pock-like lesions shown by SH79-1 web. Electron microscopy revealed poxvirus infection only in CAM inoculated with SH79-1 web.

Chorio-allantoic membranes inoculated with homogenized webs from either SH79-1, SH79-2 or SH77-21, were homogenized and passaged three times in CAM. Pock-

like lesions were not produced on passage and poxvirus infection was not detected by electron microscopy. Similarly pock-like lesions and poxvirus infection were not observed in CAM of shearwater and gull eggs inoculated with either homogenized SH79-1 web, or CAM from hens' eggs inoculated with SH79-1 web.

Attempts to infect chick embryo cell cultures and shearwater lung and web explant cell cultures with the poxvirus of SH79-1 web were unsuccessful.

#### Inoculation of shearwater webs

Two shearwater nestlings were inoculated with a clarified homogenate of SH79-1 web, two with homogenized SH79-1 web-inoculated CAM, and two controls with PBSA, and then kept under observation for 34 days. The webs of control birds appeared normal whereas the remainder were inflamed on the day following inoculation. This inflammation disappeared except for one bird, inoculated with SH79-1 web, that had a reddened area with slight inflammation on the right web for 19 days after inoculation. None of the birds developed lesions similar to those described for birds observed in the field.

#### Electron microscopy

Virions showing typical poxvirus morphology were concentrated within large inclusions in web lesions (Fig. 2) although single particles were observed, sometimes scattered among collagen fibers. The inclusions were often encapsulated by a layer of electron dense necrotic tissue and in some cases appeared to be detaching from the surrounding tissue (Fig. 2). Mature virions were generally concentrated in the central area of the inclusion bodies whereas particles adjacent to the outer edge appeared in various stages of maturation and occasionally virions were observed budding into vacuoles.

Poxvirus particles also were observed

concentrated within the pock-like lesions of CAM inoculated with SH79-1 web. A few single particles were observed scattered throughout the CAM intercellular spaces. Unlike the inclusions in shearwater webs, they contained only apparently mature virions and were not 'encapsulated' although the inclusions were well delineated within the CAM.

#### DISCUSSION

Puffinosis occurs as an epizootic affecting nestling shearwaters at the time of fledging, and when they have been deserted by their parents (Dane, 1948; Nuttall et al., 1982). During studies on the etiology of puffinosis, poxvirus infection was detected in a full-grown shearwater found in April 1979 and showing clinical signs of the disease. However, examination of the webs of eight diseased fledglings collected during the puffinosis epizootic later that year, and the webs of 17 diseased fledglings collected in other years, did not reveal the presence of poxviruses. Poxvirus infection was detected in a further five full-grown shearwaters, but none of these showed clinical signs of puffinosis. In particular, the lesions on their webs were quite unlike the blisters associated with puffinosis. The results indicated that poxviruses are not involved in the etiology of puffinosis. This conclusion is supported by two additional field observations. First, the prevalence of birds with lesions was approximately the same in different areas of Skomer Island, regardless of whether they were areas where puffinosis occurs. Secondly, four birds which survived puffinosis as fledglings did not have lesions when recaptured as 2- or 3-yr-olds, and three birds with lesions as 3-yr-olds were apparently healthy and without lesions when handled as fledglings (Brooke, unpubl. data).

Previous attempts to determine the cause of puffinosis succeeded in isolating an infectious agent that, according to fil-

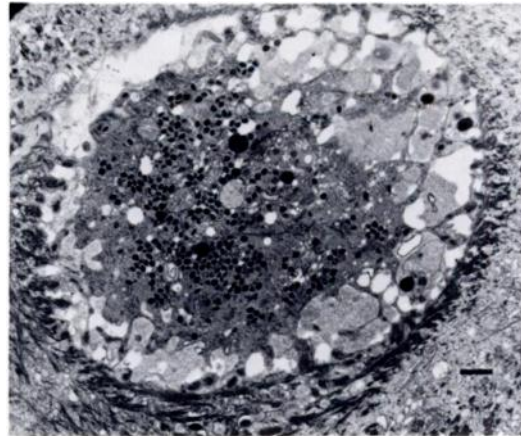


FIGURE 2. Electron micrograph of web lesion of a Manx shearwater showing inclusion body containing poxvirus. Bar = 1  $\mu$ m.

tration studies, was 20 to 30 nm in diameter, and that replicated in the CAM of embryonated hens' eggs with the formation of intracytoplasmic eosinophilic inclusion bodies (Miles and Stoker, 1948; Stoker and Miles, 1953). The authors considered that, although the formation of 'vesicles' and inclusion bodies was characteristic of poxvirus infection, the apparent size of the agent precluded poxvirus from being the isolated agent.

Attempts to isolate poxvirus by inoculation of CAM and cell cultures were unsuccessful. Although the virus replicated when first inoculated onto CAM of hens' eggs, there was no evidence of replication on passage. Miles and Stoker (1953), in contrast, were able to passage their isolate 40 times in CAM. All subsequent studies on poxvirus infection of shearwaters therefore relied on detection of virus by electron microscopic examination of web samples.

The morphology of the shearwater virus was typical of members of the poxviridae. However, the inclusion bodies observed in shearwater webs were unusual in that they comprised both mature and immature virions. In contrast, in infected

CAM, inclusion bodies comprised only mature virions and were similar to the Type A inclusion bodies (Bollinger bodies) observed for other poxvirus infections (Purcell et al., 1972; Thiele et al., 1979). Samples of lesions from the webs of five birds revealed poxvirus infection in four. Poxvirus may not have been detected in the fifth sample because it contained very little material in comparison to the others. The high proportion of poxvirus in these lesions indicates that they resulted from infection with the virus, although attempts to reproduce the lesions by experimental infection were unsuccessful. If the lesions were caused by poxviruses, the prevalence of poxvirus infection among full-grown birds lay between 1 and 4%.

Only one shearwater (SH79-1) infected by poxvirus showed clinical signs although diphtheritic poxvirus lesions were not observed during autopsy. None of the other birds with poxvirus infections were necropsied because they appeared healthy, and one bird found with a lesion in July 1983 had normal webs when next caught in June 1984. The clinical signs shown by SH79-1 may have been due to puffinosis, and infection by poxvirus only incidental. In wild bird species poxviruses generally cause a mild, self-limiting disease (Karstad, 1971) unless there is heavy infection of the head (Davidson et al., 1980; Wingate et al., 1980). The apparent prevalence of poxvirus infection in full-grown birds, and evidence of the virus in four different years, suggests that poxviruses are endemic among the shearwater population of Skomer Island. The lower prevalence of lesions in birds caught in March compared with mid-summer may reflect a higher level of immunity to poxvirus infection in the older population of March-caught birds.

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