Covert iridovirus infection of blackfly larvae

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SUMMARY

Iridovirus infections are typically isolated at extremely low frequencies from invertebrate species in moist or aquatic habitats. Crystalline arrangements of virus particles in heavily infected tissues cause an iridescent hue obvious to the naked eye; diagnosis of iridovirus infection has traditionally occurred when the host appeared bright lilac or blue. Evidence is presented here to show that there are two forms of iridovirus infection in larval blackfly (Diptera: Simuliidae) populations: (i) the classical patent form which causes larvae to develop blue iridescence before death; and (ii) a covert (inapparent) form which is not lethal. Patent infections are extremely rare in *Simulium* populations from the River Ystwyth, Wales (*ca.* 1 in 10^6). However, a large proportion of the population harbours the disease without obvious symptoms. Covert iridovirus infection was shown by using two techniques. First, tissue homogenates from 8 out of 30 apparently healthy *Simulium* larvae produced patent iridovirus infections when injected into a permissive lepidopteran host species. The DNA restriction profiles from these covert isolates showed very close similarities in fragment pattern and variability to isolates causing patent infections in the same River Ystwyth populations. Second, the presence of iridovirus DNA in host tissues was confirmed by using the polymerase chain reaction (PCR) targeted at the iridovirus major structural protein gene. The frequency of PCR-positive larvae taken from the River Ystwyth in April 1992 varied between 17% and 37%, depending on site.

1. INTRODUCTION

There can be no pathogens more attractive to the naked eye than iridoviruses, for they endow their victims with a beautiful, jewel-like opalescence. Iridoviruses (IVs) are non-occluded, icosahedral particles, approximately 130 nm in diameter, containing double-stranded DNA which assemble in host cytoplasm. Iridoviruses have been isolated from over 20 invertebrate species including crustaceans, nematodes, annelid worms, but most commonly from insects (Kelly 1985). The particles arrange themselves into regular crystalline patterns in host cells. In heavily infected tissues, light striking these viral arrays is subject to interference in the form of Bragg reflections resulting in an iridescent blue, green or lilac hue. This dramatic colour change has historically been the diagnostic characteristic for determining whether or not an insect harbours an iridovirus infection. The frequency of patent iridovirus infections in invertebrate populations is, however, often remarkably low. Most observations report a single or very few patently infected individuals amongst many hundreds or thousands of apparently healthy conspecifics (see, for example, Batson *et al.* 1976). Higher frequencies of patent infections have been described (see, for example, Boucias *et al.* 1987) but ecological studies on iridovirus–host population dynamics are limited to that of Grosholz (1992), who examined the effect of food competition on infection levels in isopod populations. The route of infection remains uncertain for all the iridoviruses, but this is probably a consequence of using iridescence as the diagnostic indicator of infection. The consumption of large quantities of virus during acts of cannibalism towards infected conspecifics (Grosholz 1992), or entry via wounds or cuticular abrasions (as particles show high infectivity when injected), have been suggested as routes of infection. Nematode parasites with the ability to act as vectors of iridovirus infections have been considered as agents of transmission for certain species (Hess & Poinar 1985; Ward & Kalmakoff 1991). Iridoviruses and their larger counterparts, the Chloridoviruses, are classified by host and number according to the sequence in which they were discovered as proposed by Tinsley & Kelly (1970).

Iridoviruses have been identified from the larvae of six species of blackfly (Diptera: Simuliidae) worldwide. Only two of the isolates, however, have been retained for study; the first, iridovirus type 22 (IV22), was isolated from blackfly larvae in the River Ystwyth, Wales in the early 1970s (Batson *et al.* 1976). The second isolate, from *Simulium vittatum*, in Canada, was described recently by Erlandson & Mason (1990), who reported distinct differences between their material and IV22 based on polypeptide characterization and DNA restriction fragment profiles. In the case of IV22, a total of 12 patently infected larvae were found during a two-year study in which some 6 million to 10 million larvae were examined (Batson *et al.* 1976; Kelly *et al.* 1978). Subsequent laboratory studies have considered *in vitro* replication mechanisms and serology (Kelly 1976, 1980; Brown *et al.* 1977), DNA restriction fragment profiles (Hibbin & Kelly 1981), infectivity to other dipteran species (Tesh & Andreadis 1992) and the characterization of the major capsid protein gene (Cameron 1990). There are no data on the ecology or

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host relationships of either isolate. Recently, Williams & Cory (1993) observed marked variation in eight iridovirus isolates causing patent infection in individual *Simulium* larvae in the River Ystwyth. All the isolates had different restriction fragment profiles, which were similar but never identical to IV22.

This study describes a covert iridovirus infection within populations of blackfly larvae from the River Ystwyth, Wales, at one moment in time (April 1992). Infected larvae appear completely devoid of the characteristic iridescent hue, but several techniques have established that a large proportion of the host population harbours this infection. The presence of iridovirus in host tissues was shown indirectly by infection of a permissive lepidopteran host, and directly by using the polymerase chain reaction (PCR) targeted at the major structural protein gene.

2. METHODS

Blackfly larvae were collected from stones in fast-flowing sections of the River Ystwyth on two occasions. On 26 March and 22 April 1992, larvae were taken from Blaenycwm (Ordnance Survey map 135, grid reference 826 755), Abermagwr (reference 667 729) and Pentre-llyn (reference 619 757) (approximately 32 km, 13 km and 7 km from the river estuary, respectively). The water temperatures were 6.7 °C and 11.5 °C in March and April, respectively, and water velocity ranged from 0.9 m s^{-1} to 1.4 m s^{-1} depending on site. A standard antibiotic solution (10 mg ml^{-1} Streptomycin and 10000 iu ml^{-1} Penicillin) was used at different dilutions for larval preservation and to prevent bacterial contamination in subsequent laboratory procedures. Larvae were placed individually in 0.2 × antibiotic solution and, once returned to the laboratory, were frozen until required. Larvae destined to be lab-reared were kept alive in large numbers in polythene bags filled with river water during the journey back to the laboratory.

(a) Laboratory rearing of simuliid larvae

Larvae taken from the River Ystwyth were reared through to adulthood in the laboratory at ambient temperature by using the following apparatus: a glass tube of 300 mm × 50 mm internal diameter with a rubber bung at one end through which a drainage tube and a syringe needle were passed. The needle was attached to an air supply from a standard aquarium air pump, which produced aeration and water turbulence in the tube. The water was replaced daily with clean tap water and the larvae were fed ad libitum with ground tropical fish food (TetraMin Flake Food). Larvae readily attached to a glass rod placed in the water which could be periodically removed and larvae examined for signs of iridescence.

(b) Diagnosis of iridovirus infection using Galleria mellonella

The greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), is highly permissive to many iridovirus infections, including IV22, and has regularly been used to 'bulk-up' viruses for experimentation (Ward & Kalmakoff 1991). An attempt was made to show that the viral entities within apparently healthy simuliid larvae from the River Ystwyth were capable of causing overt disease in *G. mellonella*. Initially, 30 *Simulium* larvae with a normal, healthy appearance, from

the April collection at the central site of Abermagwr, were homogenized individually in Eppendorf tubes with 200 μl of sterile TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Each tube was then spun in a microcentrifuge at 1500 g for 5 min to pellet debris, and the supernatant transferred to a fresh tube. This in turn was spun at 10000 g for 15 min to pellet any virus particles present. The remaining supernatant was discarded and the pellet, which never displayed the slightest iridescence, was resuspended in 120 μl of 1 × antibiotic solution: 10 μl of this suspension was injected into the haemocoel of early third instar *G. mellonella* larvae which were subsequently kept on artificial diet at room temperature and checked for iridescence at regular intervals thereafter. If no symptoms of infection had appeared at 3 weeks post-injection, the larvae were considered uninfected and were discarded. Control larvae (mostly *S. equinum* and *S. pseudequinum*) from the River Cherwell, Oxfordshire, were treated in the same way. Observations of larvae at this site had failed to reveal any IV infections during a detailed two-year study (T. Williams, unpublished data). River water from the Ystwyth was also injected into *G. mellonella*: 200 μl of river water was mixed with 200 μl of 1 × antibiotic solution, and 10 μl injected into each larva as described above.

The same procedure was also done using groups of ten apparently healthy larvae from each site collected in March or April and homogenized together as pooled samples. As before, control larvae from the River Cherwell were treated in the same way.

Those *Galleria* larvae injected with simuliid larval extracts which developed an obvious opalescence were chilled until death and homogenized in 1–2 ml of sterile TE buffer. Debris was pelleted at 1500 g, and the supernatant spun at 10000 g for 15 min to produce a brilliantly iridescent pellet of nearly pure virus. The viral pellet was resuspended in sterile TE buffer, a portion taken for DNA extraction, and the remainder stored at −20 °C.

Extraction of viral DNA from *Galleria*-grown virus was achieved by adding 400 μl of the partly purified virus suspension, to 600 μl of sterile TE buffer, 0.1% sodium dodecyl sulphate (SDS) and 100 μg of Proteinase K, and incubation for 18 h at 37 °C. After this period, remaining viral particles were pelleted and DNA extracted by treatment with phenol, phenol-chloroform, and chloroform + isoamyl alcohol (25:1). DNA was then dialysed in three changes of TE buffer at 4 °C overnight, aliquoted and stored at −20 °C.

DNA extracted from *Galleria*-grown virus was characterized by restriction enzyme digestion with *Hin*d III (Boehringer Mannheim, Germany) following the recommended protocols. Restriction fragments were separated by electrophoresis in 0.6% agarose overnight at 30 V, in TBE buffer comprising (in millimoles per litre): 89 Tris-borate, 89 boric acid, 2 EDTA, pH 8 containing 0.3 mg l^{-1} ethidium bromide. DNA fragments were visualised and photographed in UV light at 312 nm.

(c) Detection of covert infection by PCR

(i) *Extraction of DNA from blackfly larvae.* The PCR reaction is highly sensitive to environmental contamination, especially from organic matter. To overcome this problem, the head capsule and gut of each simuliid larva destined for PCR was dissected out in filtered, sterilized water with the aid of a binocular microscope. All the water used for the following steps was deionized, filtered through a 0.45 μm filter, autoclaved, filtered again through a 0.2 μm filter and re-autoclaved in 15 ml bijou tubes. Larvae were transferred individually to sterile Eppendorf tubes containing 200 μl of water and 0.5% Triton X-100. Larvae were then homogenized within the Eppendorf tube by using a sterile

1 ml pipette tip and heated to 100 °C for 5 min to eliminate DNase activity. Tubes were spun at 1500 *g* for 5 min to pellet debris, and the DNA was purified by using a resin spin column followed by an isopropanol wash (Promega Magic DNA clean-up). DNA was eluted from the column in 30 μl of water and stored at −20 °C. The concentration of a subsample of the DNAs was determined in a spectrophotometer at 260 nm. The extracted DNA (300–400 ng in 1 μl) was then used for the initial PCR amplification described below. Larval DNA from uninfected (River Cherwell, Oxford) simuliids, salmon sperm DNA and water were subject to identical purification procedures and were routinely run as negative controls alongside all River Ystwyth larval samples.

(ii) *Construction of primer sequences.* The PCR reaction involved two steps and a nested set of primers. Initially, an outer pair of primers targeted at the major structural protein (MSP) gene were used to produce a fragment 816 bases in length. These outer primers were 25 and 22 bases long, and were designed to hybridize at base number 679–704 and 1495–1473 (5′ to 3′) of the MSP gene sequence given by Cameron (1990). A second PCR reaction was then done using DNA amplified by the outer primers as the template. This second reaction required inner primers designed to hybridize at base number 733–753 and 1452–1431, and produced a fragment 719 bases long from within the 816 base fragment amplified in the first reaction. The ability of the primers to recognize the covert iridovirus isolates (grown in *G. mellonella*) was tested by amplification of approximately 400 pg of purified DNA from each isolate.

(iii) *Details of the PCR reaction.* Each reaction mixture contained the following additions, to produce a total volume of 50 μl: 42 μl water, 5 μl 10 × Taq buffer (supplied with Taq Polymerase XL by Northumbria Biologicals Ltd, U.K.), 120 ng of each primer, 1.5–2.0 units of Taq Polymerase XL (5 units per microlitre, NBL) and 1 μl of purified simuliid DNA (*ca.* 300 ng). This mixture was overlaid with 50 μl of liquid paraffin to eliminate evaporation and condensation.

The first reaction step involved the outer primers which had calculated annealing temperatures (determined by primer length and G + C content) of 70 °C and 72 °C. The program ran 40 cycles of: 95 °C for 1 min, 69 °C for 1.5 min, 72 °C for 1 min. The second reaction step involved the inner primers which had annealing temperatures calculated at 62 °C and 64 °C. The program ran 40 cycles of: 95 °C for 1 min, 61 °C for 1.5 min, 72 °C for 1 min.

(iv) *Visualisation and confirmation of PCR product.* DNA products produced by PCR were visualised by 0.6% agarose gel electrophoresis in TBE buffer with ethidium bromide, as described above. The fragment produced by the second step reaction was used as the criterion for diagnosing iridovirus infection. The identity of the 719 base fragment was confirmed by using Xho I which cuts at the 5′C↓TCGAG sequence located at base number 1205–1210 on the MSP sequence (Cameron 1990) to produce fragments of 472 and 247 bases in length. These fragments were sized by electrophoresis in 0.6% agarose alongside lambda DNA cut with Pst I which gave bands of the appropriate sizes. PCR reactions were replicated twice for negative samples, and PCR-positive samples were subject to at least one confirmation.

3. RESULTS

The dominant species present in the River Ystwyth samples at the time of sampling was *Simulium variagatum* (62%), followed by *S. reptans* (27%) and small numbers of *S. agyreatum*, *S. intermedium* and the *S. aureum*

group. Larvae were selected randomly for the injection and PCR procedures, but the majority must have been *S. variagatum*.

(a) *Laboratory rearing of simuliid larvae*

Survival of larvae in the laboratory was high (*ca.* 70%) and, despite rearing many hundreds of larvae to pupation and adulthood in the laboratory, no cases of patent iridovirus infection were observed. This clearly shows that the infections described below were truly covert, and not simply an early stage of infection which would develop into patent infections later in the development of the larvae.

(b) *Development of covert iridoviruses in Galleria mellonella*

(i) *From individual simuliid larvae.* Of the 30 individual simuliid larval extracts (from larvae taken at Abermagwr), eight produced patent infections in *G. mellonella* larvae. When the iridovirus from each infected *Galleria* was isolated and characterized by restriction with *Hind* III, all the isolates appeared different from one another, although their common similarity to IV22 was obvious, especially in the conserved fragment at approximately 6.5 kilobase pairs (figure 1). All the remaining *Galleria* larvae proved negative for patent
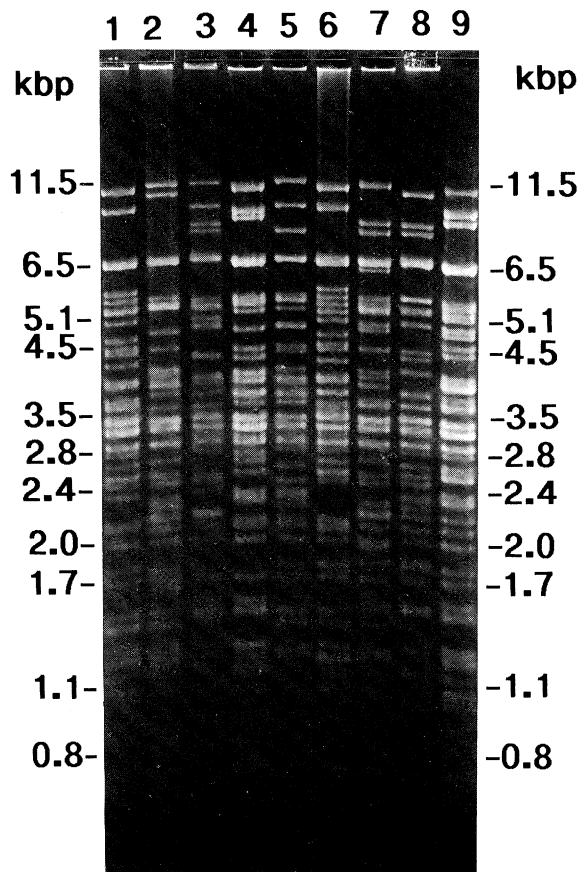

Figure 1. *Hind* III digests of iridovirus DNA produced following injection of individual apparently healthy *Simulium* larvae into *Galleria mellonella*. Lanes 1–8, patent infections caused by larvae form Abermagwr, April 1992; lane 9, IV22. All sizes in kilobase pairs, 0.6% agarose gel.
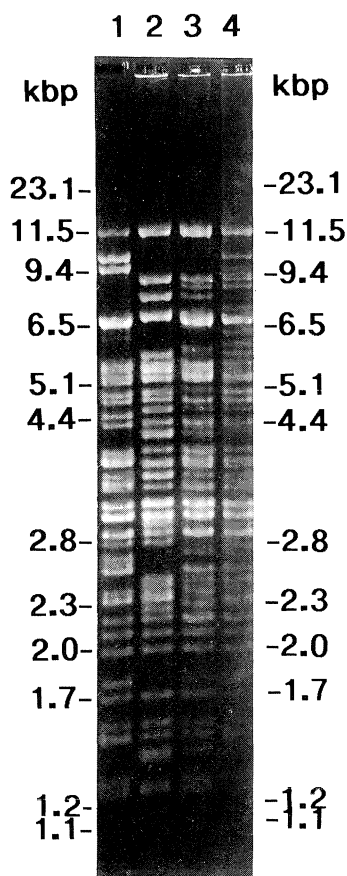
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Figure 2. *Hin*d III digests of viral DNA produced following injection of pooled samples of ten *Simulium* larvae from different sites along the River Ystwyth into *Galleria mellonella*. Lane 1, IV22; lane 2, Blaenycwm (April); lane 3, Abermagwr (March); lane 4, Pentre-llyn (April). All sizes in kilobase pairs 0.6% agarose gel.

infection when homogenized and centrifuged to check for the presence of an iridescent pellet.

(ii) *From pooled groups of simuliid larvae.* Following injection of pooled extracts of simuliid larvae, all the *G. mellonella* developed patent iridovirus infections. When viral DNA from these *Galleria* was characterized by using *Hin*d III, isolates were again shown to be highly variable in the frequency and distribution of their restriction sites, but with a conserved fragment at approximately 6.5 kilobase pairs as before (figure 2). In addition, a very large number of submolar fragments were evident. This suggests that pooled simuliid extracts produced profiles characteristic of mixed infections.

(c) PCR detection of iridovirus MSP gene in simuliid tissues

Purified DNA from all the covert isolates (produced in the *Galleria* bioassay) produced clear amplification products of the predicted size, all of which were cleaved by Xho I at the predicted site, confirming that the primer sequences were suitable for detection of MSP gene sequences in this system. The frequency of PCR-positive samples from each site on the River Ystwyth was 17% (5 out of 30) for the upstream site Blaenycwm,

37% (11 out of 30) for the central site of Abermagwr, and 23% (7 out of 30) for the downstream site of Pentre-llyn.

Calibration of the sensitivity of the PCR reaction under this purification régime and reaction conditions was done by the addition of an estimated 10^1–10^6 copies of IV22 DNA to control larvae before purification. The level of sensitivity for this method lay around 10^3 gene copies per larva.

4. DISCUSSION

PCR is a uniquely sensitive method for detecting very small numbers of specific DNA fragments present in material of environmental, forensic or laboratory origins. As such, it has the singular role of being a diagnostic tool with exceptional abilities to demonstrate latent and covert infections undetectable by other techniques. PCR demonstrated the presence of viral DNA within host tissues, whereas the *Galleria* bioassay suggested that the iridovirus exists in an infectious form, probably as particles, rather than in the form of naked DNA, or latent infection. The sensitivity of the PCR reaction was lower than the sensitivity normally associated with PCR reactions on clean purified DNA (as low as a single gene copy). This reflects losses occurring during the purification procedures and probable inhibition of the PCR reaction by environmental contaminants.

The significance of the observed variation in iridovirus isolates from covertly infected larvae remains unclear, although the pattern of variability is of the same type and magnitude as that seen in isolates causing patent infection in simuliid larvae from the River Ystwyth (Williams & Cory 1993). Clearly there are not simply two strains of simuliid iridoviruses, one of which causes overt disease and the other covert infection; rather, there is likely to exist some triggering factor(s), be it host mediated or environmental, which switches the common covert form into a pathogenic overt infection. In the case of baculoviruses, environmental stresses of one sort or another (food quality, pollution, temperature, heterologous viruses etc.) are usually cited as the trigger for expression of latent infections in insect populations (Entwistle & Evans 1985; Hughes *et al.* 1993). Laboratory rearing of simuliid larvae from the River Ystwyth produced no patently infected individuals, however, despite the apparent stresses associated with this activity (transportation in plastic bags, artificial diet and rearing techniques, elevated temperatures etc.). Clearly the relation between covert and patent strategies of infection merits further investigation.

The transmission cycle has only been described in detail for mosquito iridescent virus (Chloridovirus IV3). Larvae acquire infection per os by ingestion of large doses of virus during feeding upon diseased conspecific cadavers which have died shortly before pupation. The disease is not lethal in the adult, but is transmitted transovarially to the progeny wherein the disease progresses to the patent lethal state. Horizontal transmission to co-feeding conspecifics then occurs as

before, and the cycle is repeated (Linley & Nielsen 1968). For the Iridoviruses, only the isopod iridovirus has been shown to be transmitted effectively per os. This appears to be because cannibalism is a common phenomenon in high-density isopod populations (Grosholz 1992).

The effect of covert infection on simuliid populations will depend on the number of viral copies within each infected larva, the burden which parasitic DNA and protein production directed by viral genes impose upon host resources, and the overall impact of viral infection on host fitness. Whether covert infection causes a deleterious and tangible effect upon host fitness is of critical importance. For a virus which mainly exploits vertical routes of transmission, such costs would be detrimental to virus and host alike, the number of viral progeny being highly dependent upon the reproduction of the host. Intuitively, enhancement of reproduction in covertly infected hosts may be expected, or a bias in progeny sex ratio towards females if viral transmission were restricted to the egg. A pathogenic infection will never be sustained in a host population, however efficient its mechanism of vertical infection, without an element of horizontal transmission (Anderson & May 1981). Because iridescence has habitually been used as the criterion for infection by these viruses, and the frequency of this phenomenon is almost invariably low, the invertebrate iridoviruses have frequently been described as having a low capacity for horizontal transmission. If this criterion for infection is discarded, however, as this study suggests it should be, the infectious properties of many iridescent viruses may be substantial.

Interest in biocontrol agents for blackflies reflects the disease vector status of these haematophagous Diptera. For the reason of infectivity (above), iridoviruses have not been seriously considered as biocontrol agents. The finding of abundant, but apparently innocuous, covert infection warrants reassessment of these viruses, although caution is necessary while the ecological relationships of these pathogens remain so poorly understood, especially in relation to other members of the aquatic invertebrate community.

Several authors have suggested that iridovirus infections may be far more common than is suggested by the frequency of iridescent individuals (Kelly 1985; Poprawski & Yule 1990; Ward & Kalmakoff 1991), although no evidence to this affect has been published. The closest demonstration of this came from Moore (1973), who observed iridovirus particles in pooled groups of apparently healthy scarabid larvae in New Zealand pasture. Group sizes ranged between 10 and 31 larvae, and the frequency of patent infection was around 1%. Consequently it was unclear whether these inapparent infections were covert or simply an early stage of development of patent infections. Carter (1973) injected tipuliid larvae with homologous IV and was able to detect the presence of IV serologically by using a latex agglutination test several days before larvae began to iridesce. By using immunofluorescence, Tesh & Andreadis (1992) detected viral antigens in many adult mosquito tissues after injection of IV22 and, following a similar demonstration that iridovirus

could be detected in extracts from laboratory infected *Galleria mellonella* larvae, Kelly *et al.* (1978) suggested that the enzyme linked immunosorbentassay (ELISA) could be valuable in detecting IV infections in field populations with low frequencies of patent infections. Kalmakoff *et al.* (1990) mentioned sampling *Wiseana* populations in New Zealand and probing for IV DNA by using a [^{32}P]-labelled DNA probe, but without success. Recently, Glare (1992) reported observing icosahedral particles in the midgut tissues of the coleopteran *Costelytra zelandica*, a species known to suffer typically rare patent iridovirus infection.

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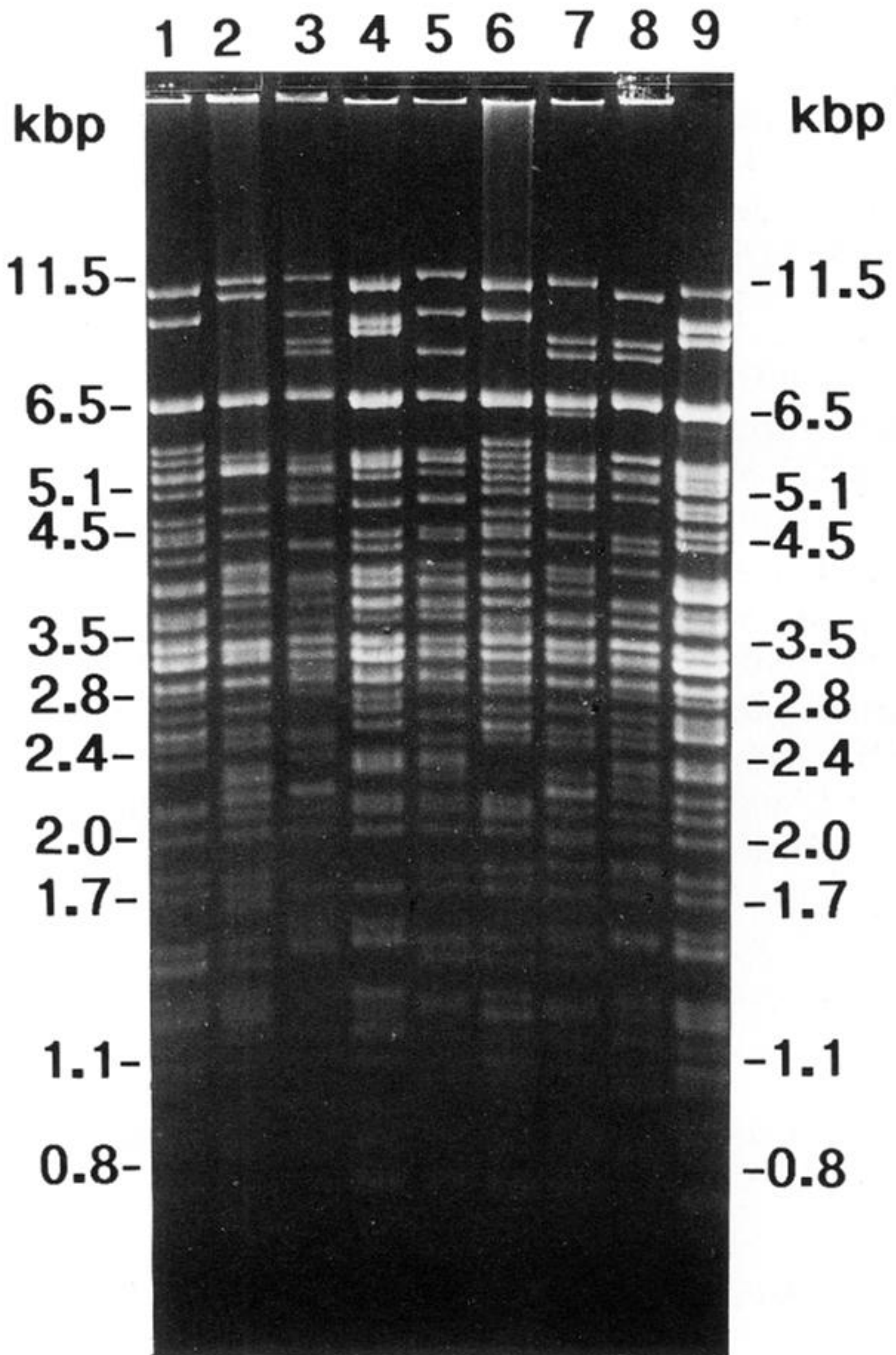
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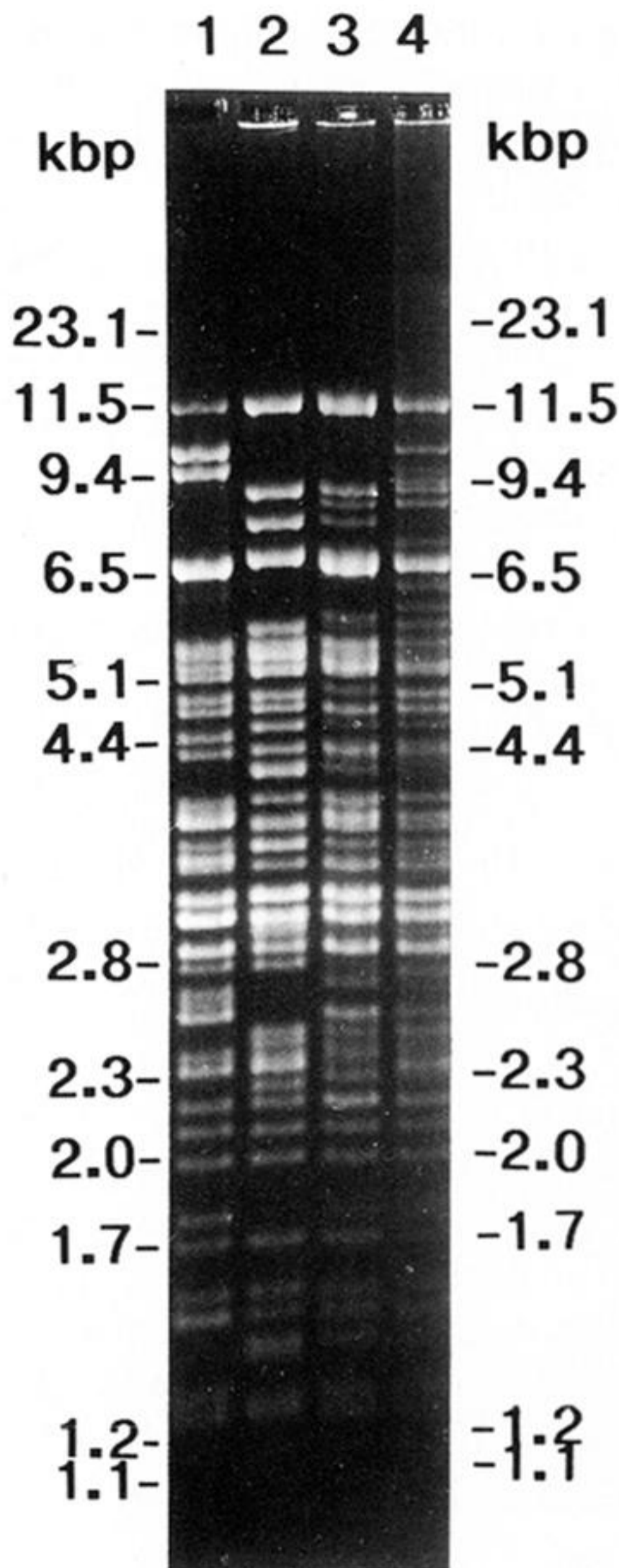
Figure 2. *Hind* III digests of viral DNA produced following injection of pooled samples of ten *Simulium* larvae from different sites along the River Ystwyth into *Galleria mellonella*. Lane 1, IV22; lane 2, Blaenycwm (April); lane 3, Abermagwr (March); lane 4, Pentre-llyn (April). All sizes in kilobase pairs 0.6% agarose gel.