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Transmission of two Australian strains of murine cytomegalovirus (MCMV) in enclosure populations of house mice (*Mus domesticus*)

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SUMMARY

To control plagues of free-living mice (*Mus domesticus*) in Australia, a recombinant murine cytomegalovirus (MCMV) expressing fertility proteins is being developed as an immunocontraceptive agent. Real-time quantitative PCR was used to monitor the transmission of two genetically variable field strains of MCMV through mouse populations after 25% of founding mice were infected with the N1 strain, followed by the G4 strain 6 weeks later. Pathogen-free wild-derived mice were released into outdoor enclosures located in northwestern Victoria (Australia). Of those mice not originally inoculated with virus, N1 DNA was detected in more than 80% of founder mice and a third of their offspring and similarly, G4 DNA was detected in 13% of founder mice and in 3% of their offspring. Thus, prior immunity to N1 did not prevent transmission of G4. This result is promising for successful transmission of an immunocontraceptive vaccine through Australian mouse populations where MCMV infection is endemic.

INTRODUCTION

House mouse (*Mus domesticus*) plagues occur in Australian grain-growing regions creating substantial socio-economic problems [1, 2]. One approach to this problem is biological control via immunocontraception [3, 4]. Interrupting normal breeding patterns is a relatively humane control method, as it avoids the morbidity associated with other strategies such as baiting. Using this approach, the animal becomes infertile by eliciting immune responses to proteins involved in reproduction.

Murine cytomegalovirus (MCMV) is being considered as an immunocontraceptive vector to control plagues of house mice in Australia [5]. To date, little is known about the epidemiology of field strains of MCMV, with most research concentrating on laboratory strains of the virus. MCMV exists naturally in Australia at a high seroprevalence in wild mouse populations (60–90% [6–8]) and in addition, individual mice can harbour more than one strain of the virus [9]. Thus, a recombinant strain of MCMV, should it be released, will be in competition with other viral strains. MCMV could prove unsuccessful as an immunocontraceptive vaccine if it cannot establish infection and transmit through populations of mice already infected with the virus.

MCMV is a large DNA virus that belongs to the *Muromegalovirus* genus of the Betaherpesvirinae subfamily, of the Herpesviridae [10]. Several factors favour the use of MCMV as a vector [5]. MCMV can persist in salivary glands for longer than a year [11],

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and resides as a latent infection in the lungs [12, 13]. Additionally, cytomegaloviruses (CMV) show strong species specificity. MCMV only replicates productively in mouse cells, while abortive replication occurs in cells from other species [14]. A recombinant MCMV is thus unlikely to pose a threat to native Australian fauna if used as an immunocontraceptive vaccine.

The MCMV isolate used as a vaccine vector should be derived from Australian wild mice, rather than a laboratory strain, as a well-established salivary gland strain may be more likely to persist and transmit between wild mice. Additionally, the use of an endemic strain as part of a released vaccine avoids the use of a laboratory strain exotic to Australia. Booth et al. [9] described two Australian strains of MCMV, designated N1 and G4, which were used in this study.

Shedding from the salivary gland is thought to be the principal means by which virus spreads through a mouse population [11]. Close contact between mice is the means by which MCMV is transmitted, as shown by housing mice in small cages in a laboratory setting [15, 16]. Preliminary investigations documented the transmission of N1 from two male or female adult BALB/c mice to eight recipient female mice after intra-peritoneal injection of 5×10^3 p.f.u. of salivary gland derived virus [G. R. Shellam and S. L. van Dommelen, unpublished observations]. G4 transmitted from wild male to wild or BALB/c female mice following the pairing of male and female mice for 21 days [15].

While these previous studies used plaque assay and ELISA, in this study we also utilized real-time quantitative PCR (qPCR) to monitor the transmission of the MCMV strains N1 and G4. The purpose of this research was to determine if immunity resulting from infection with one MCMV strain (N1) prevented transmission of a second MCMV strain (G4) within wild mouse populations released into field enclosures. We observed rapid transmission of N1 through founder mice and their offspring. There was also transmission of G4 between founder mice and to their first cohort of offspring.

METHODS

Field enclosures

The experiment was conducted in nine outdoor enclosures located at the Mallee Research Station, near Walpeup in northwestern Victoria, Australia (142·02° E, 35·08° S), a rural region, which periodically experiences plagues of house mice [2]. The construction and maintenance of these enclosures has been described [17]. Briefly, each enclosure was 15 m × 15 m, fully enclosed to prevent predation of mice, and surrounded by two zinc aluminium fences buried 700 mm below ground to prevent immigration and emigration of mice. Care was taken to remove the possibility of researcher-based transmission of MCMV [18].

Mice

Specific-pathogen free (SPF) wild-derived mice were purchased from the Animal Resources Centre (Murdoch, WA). This strain of mice were originally caesarian derived from free-living mice (*Mus domesticus*) trapped in the Murrumbidgee Irrigation Area (southern NSW) and in fields near Canberra (ACT). The founder mice were maintained as an outbred colony and mated to prevent inbreeding. Mice were 70–130 days old at the beginning of the experiment. Prior to release of mice into the enclosures, 0·2 ml of blood was obtained from the suborbital venous plexus of each mouse to confirm seronegativity to MCMV, and each was fitted with a passive integrated transponder (PIT) tag. Mice were transported from Perth to Walpeup by plane and air-conditioned vehicle following infection at the Animal Resources Centre. Infected mice were transported on a separate day to uninfected mice.

Virus

Dr A. Scalzo (Microbiology, University of Western Australia, Perth, WA) provided the MCMV isolates, N1 and G4. They were originally isolated from the salivary glands of wild mice (*Mus domesticus*) trapped at Nannup (N1), or Geraldton (G4) in Western Australia [9]. Dr D. Lang (Duke University, NC, USA) provided K181, a laboratory strain of MCMV [19, 20].

Cells and viral stock production

The M210B4 cell line was obtained from the American Type Culture Collection (ATCC). Generation of M210B4 cells for the production of tissue culture virus (TCV) stocks was as described previously [21]. Cell culture flasks (80 cm^2) containing confluent cells were infected with 1×10^5 p.f.u. of

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MCMV with 10 ml of minimal essential media (MEM) (Gibco BRL, NY, USA) with 2% fetal calf serum (FCS) under conditions of centrifugal enhancement [22, 23] at 800 ***g*** for 30 min at 25 °C. Flasks were then incubated at 37 °C with 5% CO_2 until 100% cytopathic effect was evident. Infected cells were scraped into the medium, and samples centrifuged at 11 000 ***g*** for 30 min at 4 °C. The pellet was resuspended in 5 ml of MEM with 2% FCS, frozen overnight at −70 °C and then thawed at 37 °C to release virus from cells. After thawing, the cell debris was removed by centrifugation at 300 ***g*** for 5 min at 4 °C. The supernatant was aliquoted and stored at −70 °C.

Experimental design

Farroway et al. previously described the design of this experiment [18]. Briefly, following transportation of the mice, each enclosure was seeded with a founding population of 22 (8 male, 14 female) mice. For six enclosures, three male and three female were intraperitonally inoculated with 4×10^4 p.f.u. of N1 (TCV) immediately prior to transportation. In four of these enclosures, on days 40–42 post infection (p.i.) the PIT tags were used to identify mice originally inoculated with N1. All 6 N1-inoculated mice were captured in two enclosures, five mice captured in one, and three in the final enclosure. These mice ($n = 19$) were intraperitoneally inoculated with 5×10^4 p.f.u. of G4 (TCV) and released. Two enclosure populations were used to control for researcher-based transmission. In these cages, mice were trapped and handled as for the treatment populations, but were not infected with MCMV. Mice in the central enclosure were used as controls for insect or aerial transmission. These mice were not trapped or handled until the end of the experiment.

Trapping protocol

Enclosure populations were live-trapped for three consecutive nights at weeks 4, 6, and 8 p.i., using 30 Longworth traps [24] per enclosure (5 × 6 grid). At each session weight (±0·1 g) and length (±1 mm) of mice were measured, blood collected and mice were released. The experiment was terminated at week 12 whereupon all mice were removed from the enclosures, bled, killed by cervical dislocation and the salivary glands and lungs aseptically removed and frozen at −70 °C.

ELISA

ELISA was used to monitor the seroprevalence of MCMV in enclosure populations at 0, 4, 6, 8 and 12 weeks p.i. The essential aspects of this assay have been described elsewhere [25]. Antigen was derived from virus particles and proteins obtained after ultra centrifugation of the medium of M210B4 monolayers exhibiting complete cytopathic effect induced by infection with K181. A sample was considered positive for antibodies to MCMV if the optical density at the 1/100 dilution was greater than the mean plus 3 S.D. of the negative control wells.

Plaque assay

Salivary glands were homogenized (Heidolph DIAX 300 probe, speed 3; Schwabach, Germany) in 1 ml MEM with 2% FCS, forming a 10% extract. The probe was sequentially washed with mouse osmolarity buffered saline (MOBS), ethanol, MOBS again and then MEM with 2% FCS routinely between samples to eliminate contamination. Samples were clarified by centrifugation at 1600 ***g*** for 20 min at 4 °C, and supernatant was stored at −70 °C in two 500 μl aliquots. An aliquot of each salivary gland sample was tested by plaque assay in duplicate, as described elsewhere [26], except that M210B4 cells were used. Organ weights were not measured prior to homogenization to increase the rate of processing of hundreds of samples, and to protect the viability of virus particles. Viral titres are expressed in plaque-forming units (p.f.u.) per ml of 10% extract, and are approximately tenfold less than equivalent p.f.u. per organ values. The detection limit for this assay was 100 p.f.u./ml.

Real-time qPCR

Viral DNA was extracted from 10% salivary gland extracts for the detection of N1 and/or G4 DNA by real-time qPCR. A total of 100 μl of each sample was treated at 37 °C overnight with proteinase K (10 mg/ml) and sarkosyl (10%). Two phenol:chloroform and one chloroform:isoamyl alcohol (24:1) extractions were then performed. DNA was precipitated by the addition of isopropanol, samples incubated at −70 °C for 30 min and viral DNA pellets finally resuspended in 100 μl of nuclease-free water (Promega; Madison, WI, USA). A real-time qPCR kit (Taqman Rodent GAPDH control reagents; Applied Biosystems, NJ, USA) for the detection of the

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mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for the DNA extraction procedure (Applied Biosytems). A total of 5 μl of each DNA sample was tested in a final volume of 50 μl as optimized by the manufacturer. The C_T values for the GAPDH internal controls were $21{\cdot}5 \pm 1{\cdot}9$ cycles (mean $\pm$ S.D.). The C_T value was the cycle at which a statistically significant increase in the magnitude of the fluorescent signal was first detected. Results were analysed with the GeneAmp 5700 Sequence Detection System, using the Software Version 1.3 (Applied Biosystems).

DNA samples (2·5 μl) were tested for the presence of N1 and G4 DNA. A variable site encoding a T-cell epitope in the immediate-early 1 gene (*ie1*) of N1 and G4 was chosen for amplification [27]. The primer and probes were – G4 forward primer: TACGGCTGTTT CAGATCTGAGTTT; N1 forward primer: TACGG CTGTTTCAAATCT GAGTTT; G4 and N1 reverse primer: CCTACGTAGCTCATCCAGACTCTCT; G4 probe: ACCCACACTTCATGCCCCCTAGCC-TAGG; N1 probe: ACCTAGACTTCATGCCCCC-TAATCTAGG. Primers and probes were used at concentrations of 300 nM and 125 nM respectively. The PCR reagent mix and thermal cycling conditions for all reactions were as described by the manufacturer; however, the final volume for all reactions was 25 μl, using 12·5 μl of 2× Taqman Universal PCR Mastermix. The specificity of the primers and probes for N1 and G4 detection was tested using the N1 primers and probes to test for viral DNA in samples from mice infected with G4 and vice versa. Primers and probes were specific for the detection of the appropriate viral sequences.

Negative controls were routinely included as advised by the manufacturer (Applied Biosystems), and contaminating sequences or fluorescence were not detected. Positive controls were created from N1 or G4 DNA extracted from the salivary glands of mice 18 days after intraperitoneal injection with 2×10^4 p.f.u. (TCV). The C_T values for the positive controls were routinely $22{\cdot}3 \pm 0{\cdot}6$ or $27{\cdot}8 \pm 0{\cdot}6$ (mean $\pm$ S.D.) for the detection of N1 or G4 respectively. Plasmids containing the N1 and G4 *Hin*dIII L fragments (pUC19) that encode *ie1* [28] were used to create standard curves to convert C_T scores to MCMV genome copy numbers. The Beckman DU650 spectrophotometer (at 260 nm) was used to determine the concentration (μg/ml) of plasmid DNA in prepared samples. Three samples of each plasmid were tested in triplicate. The molecular weight of the plasmid ($6{\cdot}59 \times 10^6$) and Avagadro's number ($6{\cdot}022 \times 10^{23}$) were used to compute the number of genome copies in 2·5 μl. As mouse genomic DNA and enzymes in salivary gland extracts have the capacity to alter the detection of virus, mock DNA extracts from female or male BALB/c salivary glands (6–8 weeks) were added to diluted plasmid samples to generate standard curves. The standard curves for the conversion of C_T scores to N1 or G4 genome copy numbers were $y = -1{\cdot}1182 \ln(x) + 36{\cdot}574$ ($R^2 = 0{\cdot}8732$) or $y = -1{\cdot}5458 \ln(x) + 39{\cdot}27$ ($R^2 = 0{\cdot}9512$) respectively, where y was the C_T score, and x, the genome copy number.

Statistical analyses

Two cohorts of juvenile mice were born to founder mice during the course of the experiment. Juveniles were classified into two cohorts that were a function of whole body lengths derived using frequency–length curves. Lower length cut-offs for cohort 1 (older juveniles) were determined for each enclosure and sex separately, and ranged between 71 and 75 mm. The remaining, younger juvenile mouse populations were known as cohort 2. ELISA, plaque assay and real-time qPCR were used to test the salivary glands of all founders and a subset of the juvenile population for the presence of MCMV. The required sample sizes for accurate estimation of MCMV prevalence were derived as described elsewhere [29].

Data were processed using the computer program SPSS 13.0 for Microsoft (SPSS Inc., Chicago, IL, USA). Presence or absence data were investigated by χ^2 analysis using Pearson's value. Continuous data was studied with one-way ANOVA analysis and differences elucidated using Tukey's honestly significant *post-hoc* test and the Student t test, where appropriate. Data are expressed in terms of the mean $\pm$ 1 S.E. There were no differences between replicate enclosures of each experimental treatment for any population parameter or viral measure between groups of the same sex or cohort.

RESULTS

Negative control enclosures

Mice used to control for researcher-based transmission or for insect/aerial transmission were free of MCMV by all detection methods at the conclusion of the experiment and were, therefore, not affected by

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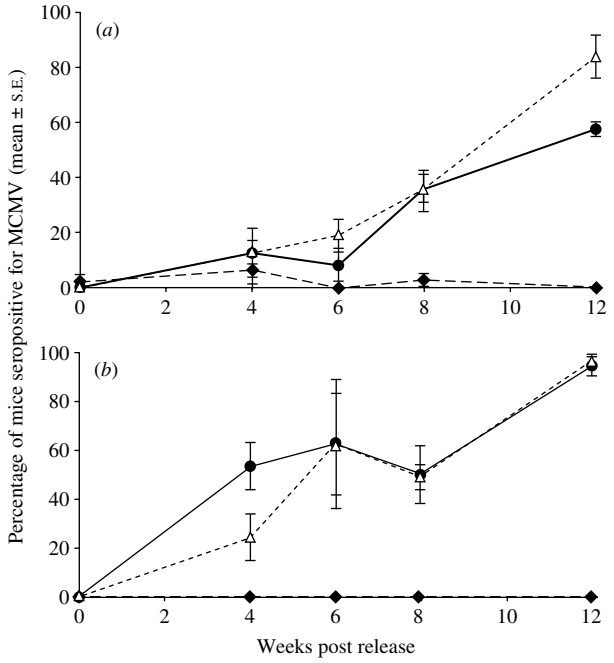

Fig. 1. The percentage of founder mice seropositive for MCMV at various times after release. Sera were collected from female (*a*) and male (*b*) mice trapped in enclosures where 25% of founder mice initially released were: (1) not infected with virus (--◆--), (2) infected with N1 only (—●—), or, (3) infected with N1, then G4, 6 weeks later (N1 + G4) (--△--).

the experimental procedures and/or transmission events occurring in surrounding enclosures. The negative control enclosures were excluded from all subsequent analyses. There was non-specific, but cross-reactive antibody detected by ELISA in mice from negative enclosures. These background levels remained low throughout the course of the experiment (Fig. 1*a*).

MCMV antibody response

At week 4, prior to the infection of mice with a second virus, there was no significant difference between antibody responses produced by N1-treated, and N1 + G4 (infected with N1, then 6 weeks later with G4)-treated enclosure populations ($P = 0{\cdot}992$, $p = 0{\cdot}319$; see Fig. 1). No juvenile animals were of a trappable age at this time post-infection.

At the conclusion of the experiment, the seroprevalence of MCMV was greater in founder male mice than founder female mice from the N1-only enclosures ($P = 5{\cdot}66$, $p = 0{\cdot}017$, D.F. = 1; see Fig. 1*a*,*b*). However, there was no difference in the seroprevalence of MCMV in male and female founder

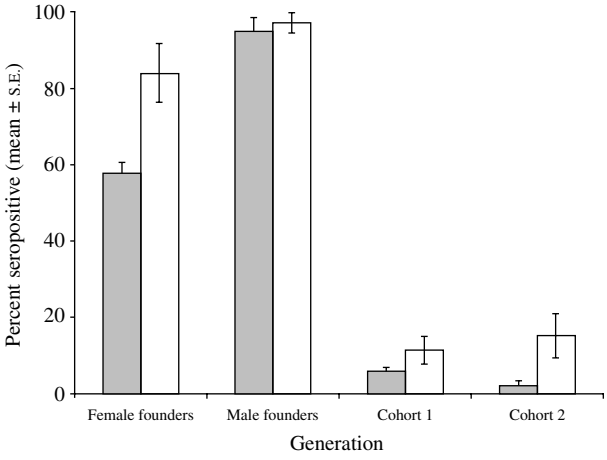

Fig. 2. The percentage of founder and offspring mice seropositive for MCMV after 12 weeks. Sera were collected from mice trapped in enclosures where 25% of founder mice initially released were: (1) not infected with virus (no virus), (2) infected with N1 only (▣), or, (3) infected with N1, then G4, 6 weeks later (N1 + G4) (□).

mice from N1 + G4 enclosures ($P = 1{\cdot}80$, $p = 0{\cdot}180$; see Fig. 2). The seroprevalence of MCMV was reduced in female founders from the N1-only enclosures in comparison to the seroprevalence observed in founder females from the N1 + G4 enclosures ($P = 5{\cdot}51$, $p = 0{\cdot}019$, D.F. = 1; see Fig. 2). There was no difference in the seroprevalence of MCMV in male founder mice trapped from either type of enclosure ($P = 0$, $p = 1$, D.F. = 1; see Fig. 2).

At the end of the experiment, the seroprevalence of MCMV in founder mice of both treatments was significantly greater than either of the offspring cohorts (N1 only: $P = 83{\cdot}5$, $p < 0{\cdot}001$, D.F. = 2; N1 + G4: $P = 153$, $p < 0{\cdot}001$, D.F. = 2; see Fig. 2). The prevalence of MCMV-specific antibodies in offspring trapped from N1 + G4 enclosures was greater than for offspring trapped from N1-only enclosures ($P = 474$, $p = 0{\cdot}03$, D.F. = 1; see Fig. 2).

Detection of infectious virus by plaque assay

Titres of infectious virus in founder mice for either treatment group were significantly greater than in either of the offspring groups (N1 only: $P = 40{\cdot}5$, $p < 0{\cdot}001$, D.F. = 2; N1 + G4: $P = 177$, $p < 0{\cdot}001$, D.F. = 2; see Fig. 3). Additionally, founder mice had significantly greater virus titres than either offspring cohort in both treatment groups (N1 only: $F = 7{\cdot}77$, $p < 0{\cdot}001$, D.F. = 154; N1 + G4: $F = 29{\cdot}7$, $p < 0{\cdot}001$, D.F. = 292).

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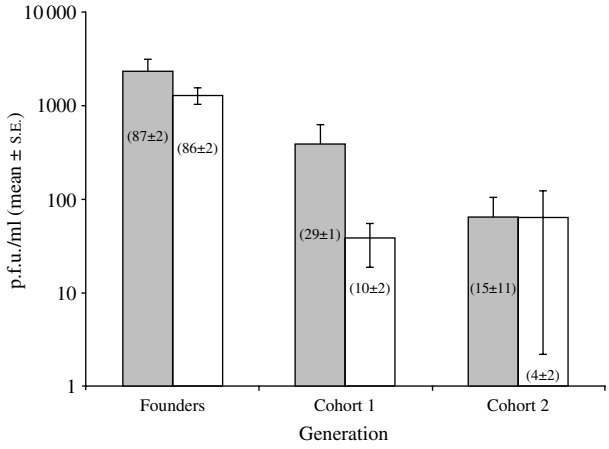

Fig. 3. MCMV titres in the salivary glands of founder and offspring mice. The viral titres were averaged for all mice tested. The percentages of founder or offspring mice with infectious virus present in salivary gland extracts are shown for each group (mean ± S.E.). Mice were trapped from enclosures where 25% of founder mice initially released were: (1) not infected with virus (no virus), (2) infected with N1 only (▩), or, (3) infected with N1, then G4, 6 weeks later (N1 + G4) (□).

There was no difference in the prevalence of infectious virus in founder mice trapped from either enclosure treatment ($P=0{\cdot}034$, $p=0{\cdot}854$, D.F. = 1; see Fig. 3). In contrast, in N1-only-treated enclosures there was a significantly greater percentage of offspring with infectious virus present in salivary glands, than observed in N1 + G4-treated enclosures ($P=21{\cdot}7$, $p<0{\cdot}001$, D.F. = 1; see Fig. 3). Although not significant, there was a trend for founder mice from enclosures with mice infected with N1 to have higher virus titres than those infected with N1 + G4 ($F=3{\cdot}00$, $p=0{\cdot}086$, D.F. = 118; see Fig. 3). A similar trend was observed during comparison of the offspring groups ($F=2{\cdot}90$, $p=0{\cdot}09$, D.F. = 98; see Fig. 3).

We detected replicating virus in the salivary glands of a small proportion of offspring (see Fig. 3). Similarly, there were low levels of seroconversion in the offspring populations. Detection of antibody specific for MCMV did not necessarily equate to high viral titre in the salivary glands of infected animals. This observation supports other studies [9].

There was no difference in the number of male or female mice with infectious MCMV present in salivary glands (N1 only: $P=0{\cdot}178$, $p=0{\cdot}673$; N1 + G4: $P=2{\cdot}42$, $p=0{\cdot}126$, data not shown). There were also no differences in MCMV titres measured in salivary gland samples taken from male or female founders (N1 only: $F=0{\cdot}126$, $p=0{\cdot}725$; N1 + G4: $F=0{\cdot}070$, $p=0{\cdot}791$) or offspring mice (data not shown).

Quantitation of N1 and G4 DNA in salivary gland samples

N1 DNA was detected in the salivary glands of >84% of founding mice, as well as 40–50% of cohort 1 offspring, and in 20% of cohort 2 offspring, as measured by real-time qPCR (see Table). There was no difference in the percentage of animals positive for N1 DNA from any generation, regardless of exposure to N1−, or N1 + G4-infected mice (founders: $P=1{\cdot}92$, $p=0{\cdot}166$; offspring cohort 1: $P=2{\cdot}38$, $p=0{\cdot}123$; offspring cohort 2: $P=0{\cdot}022$, $p=0{\cdot}882$; see Table). Thus, the prevalence of N1 was equivalent in mice of each generation between treatment groups.

The three generations of mice displayed equivalent numbers of N1 genome copy numbers for both treatments (N1 only: $F=1{\cdot}28$, $p=0{\cdot}281$, D.F. = 161; N1 + G4: $F=0{\cdot}501$, $p=0{\cdot}606$, D.F. = 292; see Table). Male and female mice also showed equivalent N1 copy numbers in both treatments (N1 only: $F=1{\cdot}11$, $p=0{\cdot}291$, D.F. = 161; N1 + G4: $F=1{\cdot}15$, $p=0{\cdot}284$, D.F. = 292). Thus, the two experimental groups displayed equivalent N1 viral genome copies for all comparisons of sex or generation between treatments.

In N1 + G4 enclosures, only 13% of founding mice, 3% of cohort 1 offspring, and no cohort 2 offspring were positive for G4 DNA in the salivary glands (Table). All founders positive for G4 were also positive for N1, however cohort 1 offspring positive for G4, were negative for N1. The number of genome copies of G4 in cohort 1 offspring was significantly greater than in founders ($t=13{\cdot}201$, $p<0{\cdot}001$, D.F. = 225). G4 was not detected in cohort 1 offspring in the enclosure where only three of the six founding mice initially infected with N1 were re-captured and re-infected with G4. There were significantly higher number of genome copies of N1 than G4 in the salivary glands of all generations ($t=2{\cdot}34$, $p=0{\cdot}01$, D.F. = 290; see Table) and there was no correlation between N1 and G4 genome copy numbers in the salivary glands of mice positive for both strains ($r^2=0{\cdot}001$).

DISCUSSION

We have shown that transmission of an MCMV strain (N1) occurs rapidly through wild mice populations held in outdoor enclosures that mimic conditions experienced by free-living mice in a mouse-plague-prone region of rural Australia. N1 transmission between founder mice increased with time, and by the

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Table. *Quantity of N1 or G4 viral DNA in salivary glands of founder and offspring mice from enclosures containing mice infected with N1, or N1 + G4*

Treatment	Generation	N1 positive* (%)	N1 genome copy number/ml (mean ± S.E.)†	G4 positive* (%)	G4 genome copy number/ml (mean ± S.E.)†
N1 only‡	Founders	83·8 ± 1·4 (n = 41)§	$5{\cdot}7 \pm 3{\cdot}2 \times 10^5$	0 ± 0 (n = 11)	0 ± 0
	Offspring (cohort 1)	50·8 ± 2·9 (n = 87)	$1{\cdot}2 \pm 0{\cdot}7 \times 10^6$	0 ± 0 (n = 8)	0 ± 0
	Offspring (cohort 2)	20·0 ± 1·2 (n = 34)	$3{\cdot}5 \pm 2{\cdot}5 \times 10^7$	0 ± 0 (n = 6)	0 ± 0
	Total	52·7 ± 0·1 (n = 162)	$2{\cdot}6 \pm 1{\cdot}1 \times 10^6$	0 ± 0 (n = 25)	0 ± 0
N1 + G4¶	Founders	89·8 ± 3·8 (n = 86)	$4{\cdot}6 \pm 1{\cdot}8 \times 10^5$	12·8 ± 5·7 (n = 86)	$1{\cdot}8 \pm 0{\cdot}7 \times 10^4$
	Offspring (cohort 1)	40·4 ± 3·1 (n = 141)	$2{\cdot}5 \pm 1{\cdot}6 \times 10^6$	2·7 ± 1·6 (n = 141)	$7{\cdot}9 \pm 5{\cdot}4 \times 10^5$
	Offspring (cohort 2)	19·9 ± 3·8 (n = 65)	$9{\cdot}5 \pm 5{\cdot}7 \times 10^5$	0 ± 0 (n = 65)	0 ± 0
	Total	51·2 ± 1·9 (n = 292)	$3{\cdot}6 \pm 0{\cdot}9 \times 10^6$	5·1 ± 2·5 (n = 292)	$2{\cdot}4 \pm 1{\cdot}8 \times 10^5$

* Mice were positive for the *ie1* DNA sequences of N1 or G4 as detected by real-time qPCR.
† Mean genome copy number/ml was calculated for only those mice positive for N1 or G4 DNA.
‡ The numbers of mice in the N1-only treatment groups at the termination of the experiment: founders (n = 41), offspring (cohort 1; n = 151), offspring (cohort 2; n = 45).
§ n = total number of mice tested, combining enclosures within the treatment.
¶ The numbers of mice in the N1 + G4 treatment groups at the termination of the experiment: founders (n = 88), offspring (cohort 1; n = 222), offspring (cohort 2; n = 76).

conclusion of the experiment, the majority of these mice were infected, as assessed by ELISA, plaque assay and real-time qPCR. This is consistent with observations that most Australian field mice are seropositive for MCMV [6–8]. The experiment also demonstrated that pre-existing immunity to MCMV (N1) did not prevent transmission of a secondary infecting strain of MCMV (G4) between founding mice and to their offspring.

N1 was detected in the salivary glands of most founding mice, and in fewer offspring, by real-time qPCR. Transmission of N1 to offspring may have been limited by age, especially within cohort 2, where mice were too young to have experienced social interactions with mice outside the nest. Additionally, maternal antibody to MCMV in colostrum and breast milk has been shown to protect neonatal mice from MCMV morbidity and mortality when intraperitoneally challenged on the first day of life and suckled until 7 days p.i. [30]. However, it is not known what impact maternal antibody has upon viral transmission.

In laboratory-based experiments, N1 and G4 were detected in the salivary glands of BALB/c mice by 14 days p.i., after intraperitoneal or intra-nasal inoculation [9] (S. Gorman, unpublished observations). Indeed, viral dissemination to salivary glands may take 7–14 days, even after experimental inoculation with a laboratory strain of MCMV [31]. Thus, N1 may not have had the opportunity to disseminate to the salivary glands of all of the young mice sampled. The experiment was limited to 12 weeks because initial predictions indicated that the number of mice present in the enclosures would increase to unmanageable levels where crowding would pose a significant threat to the health of the enclosed animals. These predictions were not correct, as mice ceased breeding after the birth of the second cohort of offspring [18].

The transmission of G4 between founding mice and to their offspring (cohort 1) was detected specifically by real-time qPCR. Transmission of G4 was also indicated by a significant increase in the seroprevalence of MCMV in founder female mice in enclosures where founder mice were infected with G4. Additionally, offspring from these enclosures were more likely to contain antibody to MCMV in sera than those from enclosures where founders were only infected with

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N1. Transmission of G4 occurred, even though by the end of the experiment 85·7% of mice initially inoculated with G4 were negative for this virus in their salivary glands by real-time qPCR.

The detection of G4 DNA in the salivary glands of the first cohort of offspring is encouraging for the successful transmission of a recombinant MCMV-based vaccine from parent to offspring where mice may possess prior immunity to MCMV. Offspring from enclosures containing mice inoculated with N1 and G4 demonstrated increased MCMV seroconversion with reduced virus titres compared to offspring from N1-only enclosures. Although less than 3% of the offspring were positive by real-time qPCR for G4 by the end of the experiment, the G4 strain transmitted at a sufficient level to alter the overall antibody status of the offspring population. G4 DNA was not detected in the youngest offspring cohort, however, there was significantly greater seroconversion in the second cohort of offspring from enclosures containing mice inoculated with N1+G4, than those from N1-only enclosures, suggesting that transmission had occurred.

G4 is transmitted from male-to-female, female-to-female or parent-to-offspring SPF wild-derived mice or BALB/c mice housed in small cages, as detected by real-time qPCR (S. Nikolovski, unpublished observations). Additionally, G4 is detected in the salivary glands and lungs of adult BALB/c mice at 12 weeks after intraperitoneal infection with 2×10^4 p.f.u. of TCV (S. Gorman, unpublished observations). These laboratory-based observations suggest that in a natural setting the G4 virus should transmit and persist in wild mice. However, measuring the transmissibility of G4 only was beyond the scope of this research.

Inoculation with N1 42 days prior to the introduction of G4 allowed for greater levels of N1 transmission and replication in the salivary glands than G4. This was reflected by the significantly higher number of genome copies of N1 than G4 in the salivary glands of all generations. All of the founding mice that were positive for G4 were also positive for N1. The introduction of G4 also increased viral-specific antibody responses. A significantly greater number of offspring from the N1+G4 enclosures were seropositive than those from the N1-only enclosures. There was also a trend for founder mice from N1+G4 enclosures to have lower virus titres than those from N1-only enclosures. These data indicate that a subsequent infection with G4 resulted in a heightened anti-MCMV immune response, which may have prevented virus dissemination and replication in the salivary glands of offspring mice.

Immunity to N1 was not sufficient to prevent transmission of G4. Indeed, 12·8% of the salivary glands of founder animals from the N1+G4 enclosures were positive for both N1 and G4 DNA. The majority of these animals were not those initially injected with virus, indicating that transmission of both strains did occur. Additionally, the introduction of G4 to enclosures did not reduce the amount or prevalence of N1 in either founder or offspring mice, as there was no correlation between N1 and G4 genome copy numbers in the salivary glands of mice positive for both strains.

In addition to the detection of viral DNA in the salivary glands, the N1 strain was detected in the lungs of a small proportion of founders and cohort 1 offspring mice for both viral treatments, and G4 was detected in the lungs of one mouse from cohort 2 offspring (data not shown). Real-time qPCR was used to detect viral DNA where there was no distinction between replicating virus and latent viral genomes in the lungs or salivary glands. Therefore, no conclusions can be made concerning the nature of viral DNA present in samples that were negative for the presence of infectious virus. Formidable logistics coordinating the sampling of hundreds of mice located at the remote outback site were not conducive for the detection of viral RNA. We could not detect transcripts of late proteins to identify whether MCMV was persisting as infectious virus at a low level, or in a latent form, as performed in previous studies [32].

In the juvenile mouse populations, real-time qPCR was the most sensitive method of detecting viral transmission. In particular, of the offspring that were positive for viral DNA in their salivary glands by real-time qPCR, only 9% and 14% of serum samples from cohort 1 and cohort 2 respectively were positive for MCMV antibody as measured by ELISA. The absence of antibody specific for MCMV in the offspring populations may have been because mice had recently acquired infection with MCMV, or that their immune systems were not fully mature and needed more time than adult mice to produce antibody.

During this experiment, mice had access to water and feed *ad libitum*, and, with the exception of infection with N1 (and later G4), were specific-pathogen free. In the wild, free-living mice find food and water for consumption, and are thus dependent upon

environment conditions that modify food abundance. If food abundance is low (for example during drought, or plague formation), malnutrition may cause immunosuppression and thus reactivation of latent virus, which is shed into secretions such as saliva, promoting viral spread. Additionally, wild mice have high pathogen loads, and infections with other organisms such as mouse hepatitis virus [8] may promote transmission of MCMV. Thus, there are a number of factors that contribute to the spread of MCMV in wild mouse populations, which were not incorporated into this experiment, and may account for lower than expected rates of MCMV transmission.

In conclusion, the success of a MCMV-based vaccine may be restricted by the presence of MCMV antibody in Australian free-living mice, however transmission may still occur despite prior immunity. Free-living mice harbour strains similar to N1 or G4, and the situation is complicated further by the presence of multiple genetic isolates of MCMV in the salivary glands [9]. A released recombinant immunocontraceptive vector may be only one of a number of MCMV strains to which an individual is exposed. Future field-based experiments should focus upon the transmission of a recombinant MCMV through populations of mice infected with multiple strains of MCMV, for time periods greater than 3 months, to focus on transmission to and within offspring generations.

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