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Virulence evolution in a host–parasite system in the absence of viral evolution

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ABSTRACT

Question: How does virulence evolve in the *Drosophila melanogaster*/sigma virus (DMelSV) system?

Organisms: *Drosophila melanogaster* (host) and DMelSV (parasite).

Empirical methods: Artificial selection on whole-carcass viral titre of infected flies, including two selection regimes (maternal and biparental transmission) and three treatments within each regime (increased titre, decreased titre, and control). The maternal transmission selection regime lasted for six generations, while the biparental transmission selection regime lasted for twelve generations. We further quantified virulence by estimating the fecundity, viability, and development time of infected flies. Finally, we sequenced virus strains at the end of selection.

Predictions and conclusions: Titre is defined here as the number of viral genomes inside a single fly, while virulence is defined as harm to host. We predicted that titre would respond to both increased and decreased selection, that virulence would evolve as a positively correlated response, and that sequence evolution in the viruses would be responsible for these changes. Titre did respond to selection in the biparental regime, although both high and control lines both demonstrated increased titre, while the titre of the low lines did not change. One component of virulence, development time, was positively correlated with titre in the biparental transmission lines (maternal transmission lines were not scored for virulence). However, we detected few (and in some cases, no) genomic changes in the virus, making viral evolution unlikely to be responsible for the response to selection and the association between development time and titre.

Keywords: host–parasite co-evolution, *Drosophila melanogaster*, DMelSV, sigma virus, rhabdovirus, virulence evolution.

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INTRODUCTION

Building a comprehensive theoretical framework for the evolution of host–parasite interactions has been a major aim of evolutionary biologists for the last 30 years (Ewald, 1983, 1987; Frank, 1996; Ebert and Bull, 2003; Alizon *et al.*, 2009). Beyond biologists' fundamental interest in studying how organisms evolve in response to selection, the study of parasite evolution is important for medicine and agriculture. From an applied perspective, understanding how changes in parasite load can drive the outcomes of disease (at both the individual and the population level) represents a fundamental step towards a better understanding of the evolution of host–parasite systems.

Two of the leading theoretical frameworks in evolutionary epidemiology – evolution due to within-host competition and evolution due to transmission–virulence trade-offs – both posit a central position of parasite load (i.e. average number/mass/volume of parasites per host) in the evolutionary dynamics of virulence (Anderson and May, 1982; Ewald, 1983; Frank, 1996). In the transmission–virulence trade-off framework, selection for increasing parasite load occurs because of correlated selection on the probability of between-host transmission, whereas in the within-host competition framework, selection for increased parasite load is driven by competitive selection on replication rate. Eventually, these selective forces are counterbalanced by a shortened infectious period caused either by increased host mortality rate or by an increased rate of parasite clearance by the immune system (Anderson and May, 1992). Selection for increased load is likely to lower host fitness (i.e. increase virulence), either directly in the case of increased host mortality or non-lethal resource loss that lowers fecundity, or indirectly through pathogenic side effects of increased host immune responses (Weiss, 2002; Day *et al.*, 2007). In the case of pathogenic side effects of host immunity, the same response that leads to increased clearance of the parasite could also decrease host fitness.

More recently, co-evolutionary theories of host–parasite evolution have emphasized that the realized parasite load emerges from an interaction between parasite characteristics such as reproduction rate and host characteristics such as resistance and tolerance (Restif and Koella, 2003; Lambrechts *et al.*, 2006; Little *et al.*, 2010). However, because of parasites' generally shorter generation time and greater manipulability, most experimental evolutionary studies have continued to focus on parasite evolution rather than host–parasite co-evolution.

Researchers have found positive relationships between titre and virulence, or between transmission and virulence, in a variety of host–parasite systems, providing support for some of the posited relationships between virulence, load, and transmission (Alizon *et al.*, 2009; Bolker *et al.*, 2010), although few systems have provided evidence of the specific trade-offs required for the trade-off theory (Froissart *et al.*, 2010; Little *et al.*, 2010). Evidence of the evolvability of parasite load, a critical component of these two theories of virulence evolution, is even more rare. Although such evolvability has been supported by observational studies of myxomatosis in European rabbits (Dwyer *et al.*, 1990) and of HIV (Fraser *et al.*, 2007), and genetic variation for parasite load is known for a variety of pathogens (Mackinnon and Read, 1999), evolvability of parasite load (especially by direct selection on load) has rarely been demonstrated experimentally in multicellular organisms (Cooper *et al.*, 2002; Mackinnon and Read, 2004; Stewart *et al.*, 2005).

Here, we test the correlated responses of changes in parasite load on parasite virulence via an artificial selection experiment, using the strictly vertically transmitted sigma virus (DMelSV, Rhabdoviridae) infecting *Drosophila melanogaster* (L'Héritier and Teissier, 1945). DMelSV has been studied for decades, since it was first identified in wild populations of

D. melanogaster as the agent causing failure of some individuals to recover after CO_2 exposure (Seecof, 1964; L'Héritier, 1970; Fleuriet, 1981). The virus is often virulent, with deleterious effects on multiple components of host fitness, including increased development time (Seecof, 1964; Fleuriet, 1996), decreased egg-to-adult viability (Wayne *et al.*, 2011), and reduced fitness in crowded conditions (Yampolsky *et al.*, 1999). Maternal transmission is likely the major route of infection by DMelSV in natural populations. Maternal transmission is more efficient than paternal transmission (i.e. close to 100% maternal transmission from females collected from the field vs. 60–65% transmission by field-collected males with uninfected laboratory females). Moreover, offspring infected by sires rarely pass the virus onto the next generation (Fleuriet, 1996; Wayne *et al.*, 2011).

DMelSV prevalence in the population we study is frequently higher than 20% (Wayne *et al.*, 2011), so matings between two infected individuals may well occur, provided that mating is not disassortative by infection status. Current data suggest that infected males enjoy higher reproductive success than uninfected males with uninfected females, and there is no indication that uninfected females are biased against infected males (Fleuriet, 1981; Rittschof *et al.*, 2013). However, data on preferences of infected females are not available. In general, data for matings where both parents are infected are sparse. For example, we do not know if there is any difference in transmission between offspring (particularly sons) infected by both parents, and offspring infected solely by dam or sire.

We subjected infected flies to artificial selection on carcass viral titre and tested for a correlated response in virulence. We used two different selection regimes, maternal transmission and biparental transmission, in each case selecting for both increased and decreased viral titre; we used the offspring of females with the highest (or lowest) titre, assayed destructively by QPCR after 5 days of egg laying, to initiate the next generation (see 'Artificial selection protocol' below). Lines from the maternal selection regime failed to respond after six generations, so we abandoned this regime but continued the biparental regime for five additional generations. In generation 12, we assayed the biparentally selected lines for virulence and sequenced the genomes of the derived viruses.

MATERIALS AND METHODS

Creation of infected lines of flies

Inoculum was prepared by homogenizing infected flies recently derived (i.e. kept in the lab for less than one year) from 12 isofemale lines collected from a natural population in Georgia (for details, see Rittschof *et al.*, 2013). The resulting supernatant was kept on ice and used to inject healthy female *D. melanogaster*, from the highly inbred line 27 [subjected to 40 generations of full sibling inbreeding prior to this experiment (Yang and Nuzhdin, 2003)]. PCR analysis verified that this line is free of *Wolbachia* (data not shown). For details of injection procedure, see Rittschof *et al.* (2013). Infection success was determined by exposing half the offspring of each injected female to CO_2 for 5 min. A fly was scored as CO_2 sensitive, and hence infected, if it failed to recuperate from CO_2 knockdown. Lines were selected for up to 10 generations, until 100% DMelSV transmission was obtained (i.e. 100% of offspring were CO_2 sensitive). Selection was used to increase transmission rates by setting up 10 full sibling pairs and testing half the offspring from each pair for CO_2 sensitivity. The other half of the offspring from the pair with the highest transmission rate were used to set up the next generation, again using 10 single brother and sister pairs per line, following Rittschof *et al.*

(2013). After eight lines reached 100% transmission, they were kept at a constant population size of five males and five females for three generations, without selection on CO_2 sensitivity, whereupon the artificial selection experiment was initiated using seven lines that were still 100% CO_2 sensitive.

Artificial selection protocol

Two selection regimes were tested: maternal transmission and biparental transmission. We expect that maternal transmission is the major route of infection in natural populations, as noted above. However, biparental transmission is interesting because it potentially allows for superinfection and thus different within-host dynamics from maternal transmission.

Throughout the experiment, flies were kept on molasses medium in polypropylene vials in an incubator (25°C, 12 hour photoperiod) on a two-week generation cycle. Because infection with DMelSV confers CO_2 sensitivity, all flies were anaesthetized by cold knockdown (immersing vials in a slurry of water and ice, and manipulating anaesthetized flies on a frozen block).

The maternal regime involved collection of a virgin female fly from the selected vial, who was then paired with an uninfected male from an independently maintained, uninfected stock of line 27 (i.e. the same line from which the infected flies were originally generated). The uninfected stock was maintained at a constant population size of five males and five females per generation throughout the experiment. For biparental transmission regime, one brother and non-virgin sister (from the same vial) were used as parents for the next generation.

At the onset of selection for both regimes, the offspring from the females with the highest and lowest viral titres out of a sample of 10 individuals per line were selected for the next generation. These two females were used to start the high titre (high treatment) and the low titre (low treatment) selection lines, respectively. After this first quantification round, the lines for the high and low treatments from the two selection regimes were propagated independently. RNA extraction and virus titre quantification were conducted simultaneously for both treatments. The seven independently generated infected lines were used for both selection regimes (biparental and maternal) treatments, for both treatments (high and low titre).

Each subsequent generation began by setting up 10 pairs of flies using the first 10 females (and males, for the biparental regime) to eclose, each in its own vial of fresh food (occasionally there were fewer flies and hence fewer vials; see Table S1 at evolutionary-ecology.com/data/2855Appendix.pdf). Because the QPCR assay requires sacrificing the females, the 10 pairs were allowed to lay eggs for 5 days prior to assay. The fixed laying time, with only a single pair of animals per vial, gave rise to similarly low densities across regimes and treatments; there were no obvious differences in fecundity for regime or treatment, though eggs were not counted at every generation. Following the laying period, the 10 females were sacrificed and assayed for virus titre. For the low treatment in both selection regimes, the vial containing eggs laid by the female with the lowest titre was retained to give rise to the next generation and the other nine vials were discarded. The same procedure was followed for the high treatment, except that the vial for the female with the highest titre was retained. After six generations of selection, we ended the maternal selection regime in part because of practical constraints (virgin collection made this regime particularly laborious), and in part because we felt that the selection response was more consistent in the biparental

regime. Selection for the biparental regime continued for 12 total generations, though one line from the low treatment was lost (415L) at generation 4. All lines had lost CO_2 sensitivity by generation 4, the first generation it was assayed in after initiating selection (G0).

Control lines were also created for all regime/treatment combinations, for each line, such that each ancestral line gave rise to three derived lines: one high, one low, and one control. To create the control lines, one of the eight vials remaining from the original 10 measured at the onset of selection (i.e. neither the highest nor the lowest) was haphazardly selected to give rise to the control line, and the titre of the founding female recorded. For each subsequent generation, the control line was propagated by setting up eight pairs of non-virgin flies in individual vials, allowing them to lay for 5 days, and then haphazardly selecting a single vial to give rise to the next generation. All eight females were measured for viral titre at every generation.

Virus titre quantification

Total RNA was extracted using TRI reagent (Sigma) in a chloroform–isoproponal extraction. The concentration of total RNA was estimated using a NanoDrop Spectrophotometer (Thermo Scientific). Viral RNA was then reverse-transcribed using a tagged sense (forward) primer of the DMelSV *N* gene [NplusTag: 5′*gcagtatcgtgagttcgagtgt*ccgatgacctgtccgtaact3′, 22 bp of non-DMelSV sequence (*italicized*) followed by 20 bp of DMelSV-specific sequence]. Each extraction product was then diluted to yield 500 ng of total RNA per reverse transcription (RT) reaction. The RNA and a variable volume of DEPC-treated water were mixed with 1 μL of NplusTag at 10 mM and 1 μL of dNTP Mix (Invitrogen) to make a total reaction volume of 13 μL. The reactions were incubated at 65°C for 5 min, followed by 1 min on ice. Next, 200 U of SuperScript III RT (Invitrogen), 10 mM DTT (Invitrogen), 40 U of RnaseOUT (Invitrogen), and 5X reverse transcriptase buffer (Invitrogen) were added (final volume 20 μL) and reactions were incubated at 55°C for 50 min, followed by enzyme inactivation at 70°C for 15 min and subsequent storage at −20°C.

Virus titre quantification was achieved using a Taqmanfi primer/probe set designed against the *N* gene in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). The forward primer matched the tag sequence from the RT primer [tag: 5′-gcagtatcgtgagttcgagtgt-3′; derived from Purcell *et al.* (2006)], while the TaqMan probe (Tprobe: 5′-catgagatggaggaactttctctccca-3′) and the reverse (R: 5′-gagtcgcagctttggagttc-3′) primer were specific to the DMelSV *N* gene. By using the tag, we could count viral genomes, as opposed to replication intermediates. Real-Time PCR reactions were performed using 2 μL of cDNA (equivalent to a quantity of 50 ng of total RNA) added to 25 μL of Real-Time PCR master mix (Applied Biosystems, Foster City, CA), 0.9 μL of both primers at 50 mM, 1.25 μL of the Taqman probe at 10 mM, and 19.95 μL of DEPC water for a total volume of 50 μL, which was split in triplicates of 15 μL each (equivalent to a quantity of 15 ng of total RNA).

Absolute quantification was used, with a five-point 10-fold serial dilution standard curve, ranging from 10^7 to 10^3. The standard was a purified PCR amplicon, using the same reverse and forward primers as in the Real-Time PCR as described above. Initial concentration of the purified amplicon was estimated using a NanoDrop Spectrophotometer (Thermo Scientific). Selection was conducted using viral copy number estimated from the standard curve. However, because of the poor quality of the standard curve used at generation 8, the raw CT value was used to select flies for this generation only.

Virulence assays

We measured four traits to test for virulence (harm to the host) as a result of infection: development time, body size, fecundity, and egg-to-adult viability. Virulence assays were performed at the last generation of selection (12) on flies from all line-by-treatment combinations (low, high, and control) for the biparental transmission selection regime only.

Development time

We measured development time by placing 10 pairs (one male and one female) of flies from each of the evolved lines in ten 200 mL bottles. Virgin offspring from the three females with the highest or the lowest virus titre, according to treatment, were used. All individuals were mated and held for 5 days prior to the assay. Each pair was placed in an inverted 200 mL bottle, which was then placed on a 35 mm Petri dish filled with fresh molasses-agar medium sprinkled with Baker's yeast. Petri dishes were changed every 12 h for 3 days, and were stored at −20°C prior to counting eggs with the aid of a dissecting microscope. Development time was estimated using eggs from replicates 1, 3, and 5 of the fecundity assay (i.e. from laying hours 12, 36, and 60; raw data are presented in Fig. S1, 2855Appendix.pdf). Within an hour of a new Petri dish being introduced to a pair of flies, 10 eggs per dish, when possible (see 2855Appendix.pdf, Table S2 shows exact sample sizes), were individually picked up using a scalpel and transferred into a new vial with fresh food. When fewer than five eggs were present, no eggs were transferred. Pupae and fly emergence surveillance began 9 days after egg transfer. The number of pupae and eclosed flies in each vial was counted every 6 h for 3 days (days 9–11), then every 24 h for 4 days (days 12–15) for each of the three replicates.

We computed the median eclosion time for each vial, and then the medians of the vial medians for each line-by-treatment combination. We subtracted 216 h (the beginning of the sampling period, and a reasonable minimum value for eclosion time) and used a mixed model to analyse the log-scaled eclosion time (log-transformation improved linearity of the pattern with respect to titre as well as the Shapiro-Wilk statistic of model residuals). We included fixed effects of treatment (i.e. selection for low vs. high titre), titre (the average virus titre actually observed in the line × treatment combination), and treatment × titre interaction (taking into account that the effect of titre could differ among treatments). We included line and line × titre as random effects, allowing for a correlation between line and line × treatment effects.

We did not directly incorporate the effects of the fecundity replicates in our statistical models, but we analysed the model residuals from our models both statistically and graphically with respect to replicate, time, and replicate × time and found no statistically or biologically interesting patterns. Fitting linear models with several random effects to small data sets is challenging (at the level of line × treatment combinations, we only had 7 lines and 6 treatment contrasts within lines), and often leads to zero estimates of random effects variances (or estimates of perfect positive or negative correlations among random effects). While we quote results from the model with all random effects (line and line × titre, allowing for correlation), we checked simpler models (including only a random effect of line, or with uncorrelated effects of line and line × titre) to ensure that our conclusions about the focal, fixed effects of titre and treatment were robust.

We used essentially the same statistical model and protocol for analyses of all fitness components; the only difference among analyses was that fitness components with normal

distributions of residuals (possibly based on transformed data) were analysed using the lme function from the R package nlme (Pinheiro and Bates, 2000), while those with non-normal distributions (fecundity and egg-to-adult viability) were analysed using the glmer function from the lme4 package (Bates *et al.*, 2013).

Body size

Body size was estimated from thorax length (Telonis-Scott *et al.*, 2005). Ten male and 10 female flies (when enough flies were present; see Table S3, 2855Appendix.pdf) from each of 10 independent vials were measured for all lines from both treatments, in generation 12 only. These flies were the animals used as parents in the fecundity experiment, and were measured at 5–7 days post eclosion. Thorax length (anterior margin of the thorax to the posterior tip of the scutellum) was measured using a dissecting microscope equipped with an ocular micrometer.

Data were analysed as described above, except for an additional fixed effect of sex. We first used a model with sex crossed with the other fixed effects, and then fitted separate models for each sex, given the observation of the typical, large difference between the sexes for size.

Fecundity

Flies used to estimate fecundity were the same animals used in the development time analysis described above. Petri dishes were changed every 12 h for 3 days, and were stored at −20°C prior to counting eggs with the aid of a dissecting microscope. Data were analysed using a Poisson GLMM with an individual-level random effect to allow for overdispersion (Elston *et al.*, 2001). Sample sizes are provided in Table S4 (see 2855Appendix.pdf).

Egg-to-adult viability

Egg-to-adult viability was estimated using the same set of eggs as for development time (protocol described in detail above; sample sizes provided in Table S5, 2855Appendix.pdf). The total number of eclosed flies was counted 18 days after egg transfer. We used a binomial GLMM with a standard logit link.

Sequencing

To identify substitutions during the course of viral evolution, we sequenced the entire DMelSV genome (12,313 bases, positions 202–12,515, using as reference the DMelSV HAP23 complete genome sequence; GenBank accession number GQ375258). Derived changes that were fixed between the high and low treatments were of interest. Thus, we sequenced two randomly selected ancestral viruses (i.e. isolated from two random females from the first generation for each set of lines; 14 genomes), as well as all evolved viruses (from the infected females with the highest and lowest virus titre, from the high and low treatments respectively, of the last generation, 13 genomes).

RNA purification and reverse transcription (RT) of the total viral genomes were performed as described above, except that for the RT we used a primer at the end of the 3′ region of the viral genome (whole1F: 5′-TAGAAGCATCCTCGGCTTTC-3′). Nineteen overlapping regions (800–950 bp length) were amplified to cover the viral genome (primers were designed using Primer3; for remaining primer sequences, see evolutionary-ecology.com/data/2855Appendix.pdf). For each sample, 1 μL of cDNA was added to 12.5 μL

PCR Master Mix (Promega), 1 μL each of reverse and forward primers (10 mM), and 9.5 μL of water, for a total volume of 25 μL. The amplification conditions were 94°C for 2 min, then 35 cycles of 94°C for 30 s, 53°C for 30 s, 68°C for 1 min, followed by a final extension at 68°C for 10 min. After verification on agarose gels, PCR products were purified using an enzymatic purification method as follows. Five microlitres of each PCR product were combined with 4.3 μL of water, 0.6 μL of Exonuclease I (Promega), 0.6 μL of Antarctic Phosphatase (Promega), and 0.5 μL of Antarctic Phosphatase Buffer (Promega), for a total volume of 11 μL. Samples were kept at 37°C for 20 min, then at 80°C for 20 min. Then, 0.5 μL of reverse or forward primer was added. All 19 overlapping regions of the 21 viruses were sequenced in both directions. Virus fragments that showed a new mutation, as well as the full genome of the virus infecting the line 440H, were sequenced twice in both directions, each time starting from an independent RT-PCR of the same RNA extraction products. All the products were sent to the DNA Analysis Facility at Yale University (Applied Biosystems 3730*xl* DNA Genetic Analyser with 96 capillaries).

Sequences are available in GenBank (accession numbers JX403908 to JX403934).

RESULTS

Response to selection on virus titre

We quantified the virus titre in 15 ng of total RNA for 10 females for every generation, as described in the Methods. The difference in virus titre between the two selection regimes increased over time, although the increase was not monotonic (Fig. 1a, b). Selection was

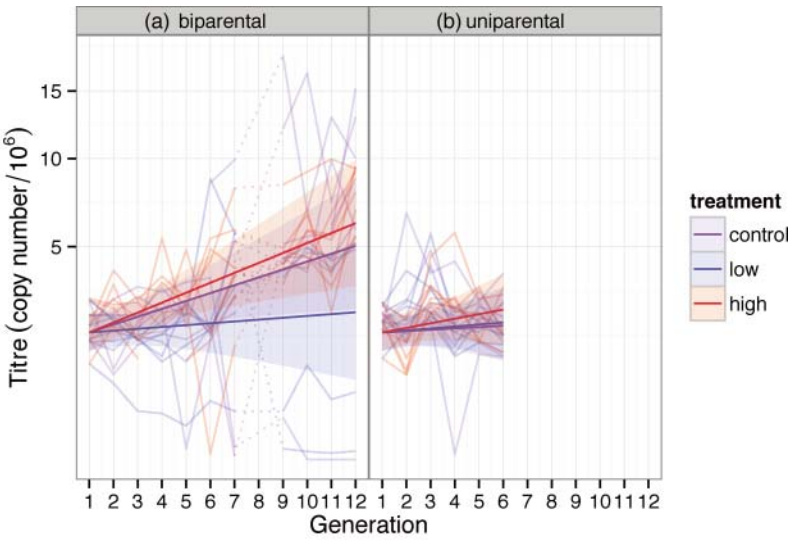

Fig. 1. Selection regimes and response of virus titre to selection: (a) biparental selection regime; (b) maternal selection regime. Virus titre is plotted on a square-root scale. Light lines show response of individual line-by-treatment combinations; dotted lines connect line-by-treatment combinations before and after generation 8 (i.e. missing data; see text). Heavy lines show fitted slopes of the linear mixed model (ribbons are 95% confidence intervals).

Table 1. Estimates of effects for titre, comparing the biparental and maternal transmission selection regimes

Source	Estimate	Standard error	Pr(> |t|)
(intercept)	1.31	0.046	$<10^{-5}$
Generation	0.062	0.015	$<10^{-5}$
Generation × selective regime	0.021	0.021	0.0808

Note: Units are differences in square root (10^6 viral genome copies).

continued for the biparental regime for 12 generations, while the maternal selection regime was stopped after six generations (Fig. 1b). We compared the two selection regimes, using all available data for both (i.e. 12 generations for biparental, six generations for maternal) following a square root transformation of the response variable. The overall slope of square-root of titre versus time averaged across treatment was statistically significant, as anticipated ($P < 0.0001$), but the difference in slopes between regimes was only weakly significant ($P = 0.08$). However, the difference in slopes is one-third of the magnitude of the average slope (see Table 1); the maternal lines had a somewhat lower slope than the biparental lines, suggestive of a weaker response to selection.

We subsequently analysed the biparental lines with respect to treatment. The high lines in the biparental selection regime increased in titre throughout the experiment, but the control lines increased in a very similar way (Fig. 1a). The overall slope of square-root of titre with respect to time was strongly positive ($P < 0.0001$), but the overall difference in slopes among treatments was not significant (generation × treatment, $P = 0.09$). More specifically, planned contrasts showed that the high and control treatments did not differ significantly ($P = 0.77$), while the low lines did differ significantly from the average of the high and control lines ($P = 0.03$). However, a permutation test using the same structure suggested that these P-values were slightly anti-conservative, and in particular that the low versus high-and-control contrast was actually only significant at a level of $P = 0.06$. We subsequently used a linear mixed model to test for differences in square-root-transformed titre at the endpoint of selection (i.e. differences in titre across treatments in generation 12), eliminating some uncertainties about outlier points and lack of normality: at the end of selection, the low versus high-and-control contrast was significant at $P = 0.04$.

Virulence assays

As a large number of virulence assays were performed, we provide a brief overview of results here and go into more detail below. We found a significant positive association between titre and development time, corresponding to approximately a 6% increase in eclosion time per 10^6 viral genomes. Despite previous work with other virus and fly strain combinations showing a cost of infection on fecundity (Wayne *et al.*, 2011) and egg-to-adult viability (Fleuriet, 1996), we detected no significant effects of treatment, titre or their interaction for either of these traits. Consistent with previous literature (Fleuriet, 1996), we did not detect any significant effects of treatment, titre or their interaction on body size.

Development time

In a model incorporating fixed effects of treatment, titre, their interaction, and random effects of line and line × treatment (see Materials and Methods), we found significant ($P = 0.049$) effects of titre equal to an approximately 6% increase in eclosion time per 10^6 copies of the virus (slope equal to 0.062 ± 0.026 log units: see Table 2, Fig. 2). The effects of treatment × titre and treatment were non-significant; control and high lines had longer eclosion times than the low lines (0.49 ± 0.22 S.E. and 0.11 ± 0.22 S.E. log units respectively), controlling for titre.

Table 2. Results of linear mixed model for development time

Source	Effect d.f.	*F*	*P*
Titre	1	5.67	0.049
Treatment	2	2.52	0.15
Titre × treatment	2	2.48	0.15

Note: Units for development time are change in model ecllosion time, in hours, by treatment or per 10^6 viral genome copies, measured from a baseline of 216 hours. Residual (denominator) degrees of freedom = 7.

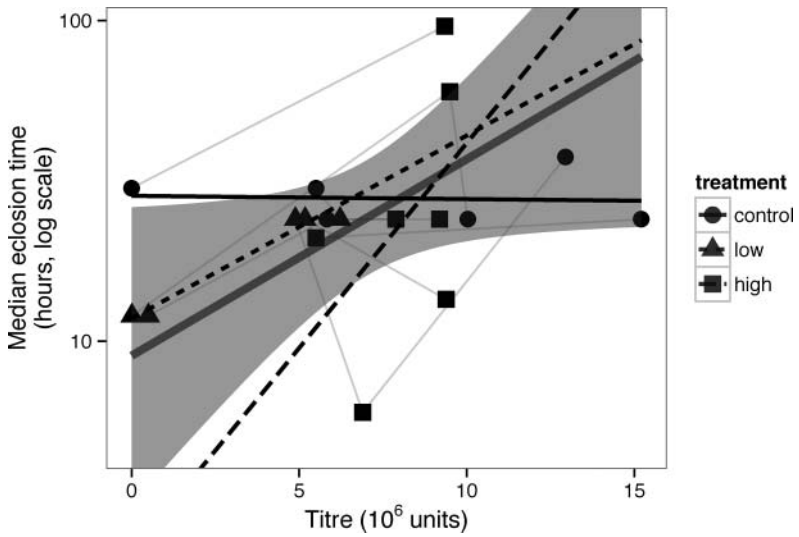

Fig. 2. Median development time, in hours, as a function of titre ($\times 10^6$) for each line-by-treatment combination. Points represent values at generation 12 from individual line × treatment combinations (● = control; ▲ = low; ■ = high); grey lines link points from the same genetic background (line). Some line-by-treatment combinations were lost, so some point types are missing for some lines. Lines show predicted titre–eclosion time relationships for each treatment; these are not significantly different from each other. The heavy line and grey ribbon show the overall titre–eclosion time relationship across all treatments, with 95% confidence intervals.

Body size

Size (as estimated by thorax length) was analysed using a linear mixed model (see Materials and Methods for details). As is typical in *D. melanogaster*, males were much smaller than females, so the two sexes were analysed separately. There were no significant relationships between size and treatment, size and titre or any interactions (Fig. S2, Tables S6–S8, 2855Appendix.pdf). The predicted average thorax width increased very slightly (from 0.88 to 0.91 mm for males and from 1.008 to 1.01 mm for females) from minimum to maximum titre.

Fecundity

The number of eggs per 12 h laying period (see Materials and Methods for detailed description of egg collection and data analysis) was analysed using a Poisson generalized linear mixed model (Table S9, 2855Appendix.pdf). There were no significant effects of treatment, titre or their interaction (Fig. S3, 2855Appendix.pdf); on average, fecundity increased by 6% (0.061 log units ± 0.048 s.e.) per 10^6 copies of the virus.

Egg-to-adult viability

Viability was calculated as the proportion of eclosed adults per vial; 5–10 eggs were used for each vial. No progeny eclosed from a large number of vials (106 vials out of a total of 203). This may have been due to stress following egg transfer to new vials; given the high failure rate, results should be interpreted with caution. For successful vials, where at least one offspring eclosed, no significant effects of titre, treatment or their interaction were found (see Fig. S4, Table S10, 2855Appendix.pdf). However, egg-to-adult viability did decrease from 0.1403% to 0.0421% for every 10^6 copies of the virus, from minimum to maximum titre.

Sequencing

Eleven derived (i.e. different from the ancestral line) substitutions were fixed between the high and low treatments across the set of six pairs of lines (Table 3; 415H is also included, though no substitutions were found relative to its ancestor). Five substitutions were synonymous and six were non-synonymous. There was no obvious clustering with respect to particular genes, or regions of the genome. Eight substitutions were in the low treatment lines, while only three were in the high treatment lines; one out of six low lines had no derived mutations, while five out of seven high lines had no derived substitutions. However, the difference in number of substitutions between treatments was not significant by a chi-squared test ($P = 0.08$).

Analyses based on parsing mutations into coding and non-coding were not attempted, given the small total number of changes. Moreover, in a virus, non-coding mutations frequently have non-trivial fitness effects, such that the difference in selection coefficients between coding and non-coding substitutions is not expected to be as drastic as in eukaryotes. Accordingly, we included the number of mutations as a covariate in the same linear model discussed earlier testing differences among treatments, but number of mutations was not significant ($P = 0.71$). Moreover, the effect size is both relatively small

Table 3. Summary of derived sequence differences after 12 generations of artificial selection (biparental transmission regime, high and low titre lines only)

Gene	N	nc	P	X	nc	M	G	L			
Position	207	279	1559	1606	3241	3479	3512	4103	4387	6091	9884
Consensus	A	T	G	A	A	A	A	G	A	C	C
239H	.	.	.	.	.	.	.	.	.	.	.
239L	.	.	.	G	G	.	.	.	G	.	.
242H	.	.	.	.	.	.	.	.	.	.	.
242L	G	.	.	.	.	.	.	.	.	.	.
325H	.	.	.	.	.	.	.	T	.	.	.
325L	.	.	.	.	.	.	.	.	A	.	
38H	.	.	T	.	.	C	.	.	.	.	.
38L	.	.	.	.	.	.	.	.	.	.	T
415H	.	.	.	.	.	.	.	.	.	.	.
438H	.	.	.	.	.	.	.	.	.	.	.
438L	.	.	.	.	.	.	.	.	.	.	.
440H	.	.	.	.	.	.	.	.	.	.	.
440L	.	G	.	.	.	.	G	.	.	.	.
Amino acid	N/S	W/L	.	E/G	Q/R	.	.	G/*	.	.	H/Y

Note: Bases that differed from the ancestral (G0) sequence of the virus for each line. Gene names are listed in the first row (nc = non-coding), positions relative to GQ375258 in the second row, and amino acid changes (where relevant) in the bottom row.

and of the opposite sign expected (although the confidence intervals are large and include positive values): −0.11 (CI: −0.67 to +0.45) square-root titre units, relative to the change between control/high and low of +0.9.

Surprisingly, for line 38, all viral sequences (ancestral and derived) had a premature stop codon at position 2754, at the beginning of the X gene. For these same four viruses, a single base insertion was also found in the X gene at position 2798.

DISCUSSION

Parasite load (viral titre) diverged between the high and low treatments in our experiment, indicating a response to selection: titre increased five-fold in the control and high treatments, and less than two-fold in the low treatment. Increased development time, a component of virulence, was associated with increased titre, as suggested previously (Gilchrist *et al.*, 2004). To our surprise, the virus evolved little or not at all during the course of the experiment; we conclude that, after considering many alternatives, response to selection is likely to be attributable to host rather than parasite evolution, even though the latter was our intended target of selection.

Non-evolutionary processes

It is possible, but we think unlikely, that the selection treatment caused titre changes via non-evolutionary mechanisms. Selection may act on titre even in the absence of (epi)genetic variation for titre in either hosts or parasites as long as (1) maternal hosts' viral titre is positively correlated with the titre they package in their eggs, and (2) vitellogenic females'

titre is positively correlated with the titre in the egg they hatched from. The first correlation, between maternal and egg titre, is plausible, although a study using endpoint dilution and subsequent infection by injection as an indicator of titre found no such relationship (Seecof, 1964). The second correlation, between egg titre and the titre of egg-laying females, also seems reasonable, but dynamical considerations make it unlikely. Suppose that some variation in titre is introduced in each generation by developmental and environmental variation (which certainly exists even in a laboratory setting, and which must be present in order to have generated among-lineage variation in titre in the first place). For a lower- or higher-than-average titre to persist from the egg stage to vitellogenesis (i.e. for titre in these two stages to be correlated), either the virus present in the egg must represent a significant fraction of the final virus load in the vitellogenic female (i.e. there is little new virus production in each generation), or the production of new virus must be proportional to the amount currently present in the system. This is true if the virus replicates exponentially within the host up until the time of vitellogenesis, but if titre quickly reaches a carrying capacity determined by host size and/or condition [as is true for DMelSV (L'Héritier, 1970)], then most of the variation in the titre contributed in the egg will be newly generated variation, and the correlation will thus be destroyed. The only biological scenario we have been able to concoct that allows for non-(epi)genetic heritability is if the virus interacts with host growth or immune processes early in development to modify resource distributions or pathogen defence (the likely determinants of the stable titre load) in the adult fly.

Viral evolution (NOT)

Viral evolution is an unlikely explanation for the observed changes in titre. In generation 12, five of seven high treatment lines had exactly the same nucleotide sequence as the ancestral viruses (i.e. viruses from the first generation of selection), yet titre was higher for these lines than in the ancestor (unfortunately, sequence data are not available for the control lines). The evolved lines 438L and 438H (derived from the same ancestral line), with identical genotypes in generation 12, diverged most in titre levels. The response to selection was greater in the high lines than the low lines (Fig. 1a), but most of the viral mutations occurred in the low lines (three in the high lines, eight in the low lines). Finally, the association between number of mutations and titre was small (actually negative, indicating that mutations decreased virus titre) and non-significant. These patterns also fail to explain the increase in titre in the high and control lines. However, we cannot definitively rule out the possibility that one of this small number of substitutions had such a large phenotypic effect as to explain some of the among-treatment divergence in titre levels.

Phenotypic or epigenetic manipulation of the host is an intriguing mode of virus-driven evolution (it is hard to categorize such processes as either 'virus evolution' or 'host evolution' because they are essentially interactions within the extended genome of the host–pathogen system). Some viruses actually encode suppressors of RNAi (Zhou and Rana, 2013), which would interfere with host defence and thus increase titre levels, but it is not obvious that DMelSV is such a virus, and given the paucity of viral substitutions, we are still left with the difficulty of explaining the among-treatment divergence. DNA methyltransferase 2 (*Dnmt2*) is a host-encoded epigenetic modifier of positive strand RNA viruses in *D. melanogaster*, but *Dnmt2* knockouts have no effect on DMelSV titre (Durdevic *et al.*, 2013). The virus might also manipulate the host epigenetically, for example by changing the histone code (Van Opdenbosch *et al.*, 2012). Such histone modifications could be heritable across

generations, and flies have homologues to the known human genes and pathways exploited by herpes virus to cause epigenetic changes (Spedale *et al.*, 2012), although their effects in response to DMelSV infection have not been studied. Again, however, one must explain how such virally induced epigenetic changes could affect the high and low lines differently given the nearly identical viral genotypes across treatments.

Host evolution

By excluding non-evolutionary and virus-evolution scenarios, the only remaining explanation for the observed response to selection is host evolution. This was not the evolutionary process we initially had in mind, and it initially seemed implausible because the treatment and control lines were initiated from highly inbred lines (meaning there would be little pre-existing genetic variation among host lines); in addition, rates of viral evolution are normally expected to be orders of magnitude greater than those of host evolution (but see below).

Could the observed effects have occurred in the complete absence of selection, i.e. purely by fixation of existing deleterious alleles via genetic drift? Our use of multiple replicate lines in all treatments makes pure genetic drift an unlikely explanation: the effective population size is the same in all treatments, so drift (whether in the host genotype or in epigenetic modifiers) cannot explain the divergence between low and control/high treatments.

However, we can invoke a combination of drift and selection to argue that the observed divergence between lines was caused by the *differential* accumulation of deleterious mutations in the host genotype. Census size and effective population size in the experimental culture decreased relative to the previous laboratory culture conditions, from vials consisting of five females and five males to a single pair of flies in each vial. Thus, we propose that drift-induced increase in the host mutation load in the control and high lines weakened host immune responses to DMelSV infection and consequently increased titre, while selection for decreased titre in the low lines worked in concert with purifying selection. This possibility could also explain the apparent trend for slower response to selection in the biparental selection regime, because effective population size was much greater in the biparental than maternal regime.

In addition to reduced effective population size, all of our experimental treatments had decreased density relative to the previous laboratory culture conditions (2 flies per vial vs. 10 flies per vial). Yampolsky *et al.* (1999) suggested that density reduction could relax selection against DMelSV (i.e. reduce virulence); they inferred this relationship from increased prevalence of DMelSV (as assayed by CO_2 sensitivity) after a switch from reduced-density (two flies per vial) to high-density (300–500 flies per vial) conditions. While our situation differs from that of Yampolsky *et al.*, their results suggest to us that reduced density could reduce the relative cost of higher titres, as well as the overall fitness costs of DMelSV.

A final, potentially important difference in culture before and after commencing artificial selection was that prior to the experiment, progeny were haphazardly selected from among those eclosing over a 14 day generation time, while during selection the first 10 females to eclose were used to seed the next vials. As our results suggest, a positive correlation between titre and development time, picking the fastest-eclosing flies might have retarded the response to selection for high titre. The relative strength of this selective filter could conceivably differ according to baseline development time and hence could accelerate the

divergence between treatments once they started to diverge in titre level and development time, although we would need a much more precise quantitative model of the effects in order to decide whether this effect would make a significant contribution to the observed divergence.

We interpret the observed increase in development time in the control/high lines as a component of virulence, i.e. as a consequence of the increase in titre. While the increase could also be driven directly by the accumulation of deleterious mutations in the host genotype, we think this is unlikely for several reasons. First, there is no main effect of treatment on development time. Second, we saw no significant between-treatment divergences in either fecundity or hatchability, although both of these traits have extremely large mutational target sizes, at least as large as for development time. Thus, unless mutations permitting viral proliferation are especially likely to be pleiotropic for development time, it seems likely that the observed increase in development time is a consequence of increases in titre.

Why did the high and control lines fail to diverge? Our only hypothesis is that despite the relaxation of selection due to reduced density and population size, selection may have imposed an upper limit on titre such that our selective regime was unable to increase titre in the high lines beyond the level experienced by the control lines (Falconer and Mackay, 1996). This explanation would require a rapid non-linear increase in the virulence as a function of titre at levels just above those observed in the control/high lines. At present, we have practically no information on the shape of the virulence–titre curve in the *Drosophila*–DMelSV system, a limitation common to most host–pathogen systems despite the importance of the shape of this curve to theoretical expectations about virulence evolution (Bolker *et al.*, 2010).

We conclude that a combination of overall relaxed selection on host response to DMelSV, combined with purifying selection for stronger host response in the low-titre treatment, is the most likely explanation for the observed changes in titre and development time under selection. We must then wonder that a dipteran would evolve faster than a virus, especially as negative-sense ssRNA viruses are expected to have a much higher mutation rate than *Drosophila melanogaster* [on the order of 10^{-4} mutations/genome/replication for viruses vs. 4×10^{-9} for *Drosophila* (Drake, 1993; Drost and Lee, 1995; Duffy *et al.*, 2008; Keightley *et al.*, 2009)]. However, DMelSV may have an anomalously low mutation rate; though its mutation rate has not been estimated, its neutral mutation rate has been estimated via neutral substitution rate at 4.6×10^{-5} substitutions per site per year (Carpenter *et al.*, 2007), an order of magnitude lower than expected. A low effective mutation rate may result from the vertically transmitted lifestyle (Duffy *et al.*, 2008). Vertically transmitted, persistent viruses in plants, which are similar to DMelSV in being present in all cell types and in being strictly vertically transmitted via the germ cells, also have low genetic variation (Roossinck, 2010; Longdon and Jiggins, 2012). Moreover, with limited mutation, repeated selection over the short parasite generation time may exhaust parasite genetic variance, which would then limit viral adaptation to an evolving host despite the relatively shorter generation time of the parasite (Gandon and Michalakis, 2002).

Follow-up experiments

Since many of our conclusions rely on incomplete evidence, it is essential to consider what further empirical observations could confirm or falsify our conclusions that host rather than virus evolution was responsible for the observed treatment responses, and that changes

in development time are caused by changes in titre. Unfortunately, the natural history of the system precludes some of the more obvious options.

- *Reciprocal infections*: To confirm or refute the argument that titre changes are caused by host rather than virus evolution, one could reciprocally infect groups of naïve flies from the same genotype with virus extracted from low versus high lines, thus determining whether differences in titre and/or development time can be recreated in a homogeneous host background. An experiment of this scale is extremely logistically challenging. Moreover, as with many experiments with an expected null result, interpretation would be difficult: negative results would be consistent with our conclusion that host rather than viral evolution occurred in our experiment, but also with many other more mundane explanations (differences between infections resulting from injection and vertical transmission, imprecise delivery of titre or incorporation of different titre into the germ cells, etc.). A positive result showing that viral effects differed in a homogeneous host population would be stronger evidence in favour of viral evolution and against host evolution, but a conclusive test would require using plaque-purified virus to rule out co-injection of some host-associated factor such as a microRNA.
- *Curing virus*: The conclusion that changes in development time are indirect physiological effects of increasing titre, rather than pleiotropic effects of the mutations that allow higher titre, could be tested by curing treatment lines of DMelSV and observing whether differences in development time persist, at least in the short term, in the absence of the virus. Unfortunately, *D. melanogaster* cannot easily be cured of DMelSV.
- *Direct manipulation of titre*: Manipulating titre directly via injecting different amounts into flies and observing its effects on development time would be a more direct test of the titre–development time link; persistence of differences in injected titre levels across generations would refute our argument against non-evolutionary changes, while an absence of effects on development time would refute the titre–development time link (although with the same caveats about negative results as described above). Such novel experimental infections would be challenging to perform, but are definitely worth investigating in the future.

In conclusion, this study provides evidence for the evolvability of parasite load and for the resulting evolution of virulence. Surprisingly, our results suggest that this relationship was not driven by the viral genome. In retrospect, it should not have been surprising that virulence could evolve without changes in the parasite – after all, virulence as defined by loss of host fitness is an emergent property of host *and* parasite. Several other experimental studies have demonstrated that traits such as virulence or transmission are the product of host and parasite genomes and their interactions [$G \times G$ interactions (e.g. Thompson and Burdon, 1992; Peever *et al.*, 2000; Carius *et al.*, 2001; Kaltz and Shykoff, 2002; Lambrechts *et al.*, 2005; Salvaudon *et al.*, 2005, 2007; Grech *et al.*, 2006; de Roode and Altizer, 2010; Bryner and Rigling, 2011)]. However, much of the previous theoretical work assuming a link between parasite reproduction and virulence has neglected host evolution (Dybdahl and Storfer, 2003; Alizon *et al.*, 2009). Co-evolutionary processes have not been well incorporated in the classical theoretical framework of evolution of parasite virulence (Restif and Koella, 2003). Indeed, it seems appropriate to consider the extended phenotype of host + parasite (Dawkins, 1982), or even host + parasite + microbiome, as a superorganism (Claverie, 2006; Forterre, 2011). Our results underscore this most fundamental tenet of parasitology, that neither parasite nor host may be considered in isolation, and highlight

the importance of considering the host as well as the parasite as we consider strategies to manage virulence.

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