

## RESEARCH ARTICLE

# A viral aphrodisiac in the cricket *Gryllus texensis*

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**ABSTRACT**

We identified the insect iridovirus IIV-6/CrIV as a pathogen of the cricket *Gryllus texensis* using electron microscopy (EM) and polymerase chain reaction (PCR) analysis. EM showed that the virus attacks the fat body, an organ important for protein production, immune function and lipid storage. During infection the fat body hypertrophied, but egg production withered, leaving the lateral oviducts empty of eggs; the females were effectively sterile. EM of the testis of infected males suggests that the testis was not invaded by the virus, although sperm taken from the spermatophores of infected males showed little or no motility. Nevertheless, males and females continued to mate when infected. In fact, infected males were quicker to court females than uninfected controls. The virus benefits from the continued sexual behaviour of its host; transmission studies show that the virus can be spread through sexual contact. Sickness behaviour, the adaptive reduction of feeding and sexual behaviour that is induced by an activated immune system, was absent in infected crickets. Total haemolymph protein was reduced, as was phenoloxidase activity, suggesting a reduction in immune protein production by the fat body. The evidence suggests that during IIV-6/CrIV infection, the immune signal(s) that induces sickness behaviour is absent. Curtailment of a host's sickness behaviour may be necessary for any pathogen that is spread by host sexual behaviour.

**KEY WORDS:** Parasitic manipulation, Sickness behaviour, Sexual behaviour, STI, Sexually transmitted infection

**INTRODUCTION**

Viruses are known manipulators of host behaviour (van Houte et al., 2013). For some viruses, certain changes in host behaviour appear to be an important element in successful viral transmission (e.g. Burand et al., 2012). In these systems, there will be intense selection pressure on the virus to manipulate its host. The existence of this evolutionary pressure provides us with the opportunity to discover how evolution has solved the neurobiological problem of behavioural control. This problem is especially interesting in the context of viruses as they tend to have small genomes, and so have a limited number of potential molecular tools.

How viruses, or any other pathogen/parasite, alter host behaviour remains poorly understood (Adamo, 2013; van Houte et al., 2013; Adamo, 2012; Lefèvre et al., 2009). Most pathogens appear to use multiple mechanisms simultaneously to alter host behaviour, as opposed to relying on a 'magic bullet' approach (Adamo, 2013). One mechanism that appears to be a common route leading to

altered host behaviour is the interruption of immune/neural communication signals in the host (Adamo, 2013).

Signalling molecules produced by the immune system (e.g. cytokines) can induce and/or suppress a wide range of host behaviours in both vertebrates (Dantzer et al., 2008) and invertebrates (Adamo, 2008). These immune-induced changes in behaviour are known collectively as 'sickness behaviours', and they help animals recover from infection (Hart, 1988). Such immune/neural connections may be especially prone to disruption by parasitic manipulators, because all parasites are under heavy selection to interfere with host immune function (e.g. by altering cytokine production). Hijacking immune/neural communication pathways may be a small evolutionary step (Adamo, 2013). In some host–pathogen systems it may be important to prevent host behavioural responses to infection (i.e. sickness behaviours) as these behaviours may reduce viral transmission and/or interfere with viral manipulation of behaviour.

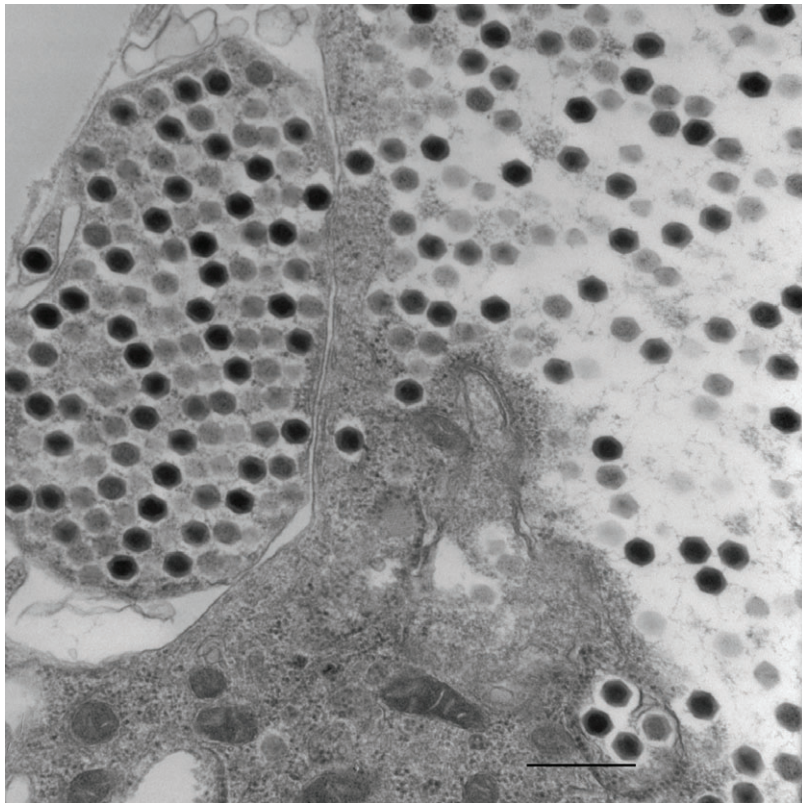
Some viruses are transmitted by sexual contact (e.g. Burand et al., 2012). Some of these viruses cause host sterility; for example, the *Helicoverpa zea* nudivirus 2 sterilizes its moth host, *Helicoverpa zea* (Burand et al., 2004). Therefore, continued sexual behaviour does not increase host fitness. It is, however, vital for viral transmission (Burand et al., 2004). Females infected with *Helicoverpa zea* nudivirus 2 show increased sexual behaviour, leading to enhanced viral transmission (Burand et al., 2005). Although the mechanistic details remain to be determined, the virus appears to use multiple methods to manipulate host sexual behaviour (Burand et al., 2005; Burand and Tan, 2006). One mechanism not yet explored in this system is the effect of the virus on host sickness behaviour. In most animals, an immune challenge results in a reduction in sexual behaviour [e.g. crickets (Jacot et al., 2004)]. Therefore, all sexually transmitted pathogens probably need to suppress the expression of host sickness behaviour in order to maintain or increase sexual behaviour in their host.

In this paper we examined the effect of an iridovirus on the cricket *Gryllus texensis* (Cade and Otte, 2000). Iridoviruses are large icosahedral, cytoplasmic DNA viruses that attack invertebrates, reptiles and amphibians (Williams et al., 2005). The viruses are probably common in field populations, and can cause epizootics (Williams et al., 2005). One type of iridovirus [IIV-6; cricket iridovirus isolate (CrIV) (Jakob et al., 2002)] attacks crickets. The fat body is the primary target (Just and Essbauer, 2001). It takes on an iridescent blue sheen, due to the large number of viral particles that are arranged in a paracrystalline array (Williams et al., 2005). The fat body is the major site of protein production in insects, as well as a storage organ for lipid (Chapman, 1998). It is also critical for disease resistance because it produces proteins vital for immune function (Chapman, 1998). The haemocytes, the immune cells of the blood, are also infected (Kleespies et al., 1999); in other words, this virus invades all major immune tissues of the cricket. Therefore, this virus is well placed to alter immune/neural communication in its host. For example, a decrease in the production of host immune proteins could suppress the expression of the host's sickness behaviour.

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Received 3 February 2014; Accepted 5 March 2014



**Fig. 1. Cytoplasm of fat body cell infected with IIV-6/CrIV virus.** The scale bar represents 500 nm.

## RESULTS

### Identity of the virus

The blue sheen of the fat body suggested the presence of an iridovirus (Williams et al., 2005). Electron microscopy (EM) confirmed the presence of a large (~150 nm) icosahedral virus (Fig. 1). The fat body cells were heavily packed with virions. Viral particles were also found in the tracheal epithelial cells and muscle. In some cells, virions formed small paracrystalline arrays. Viral particles were not found in the cells of the testis.

No other pathogens were found. Culturing of fat body or haemolymph from infected crickets ( $N=6$  females and  $N=4$  males) produced no growth on nutrient agar plates, suggesting that there were no common bacterial or fungal pathogens present.

Given the EM results, and from descriptions in the literature (e.g. Kleespies et al., 1999; Williams et al., 2005), the virus was tentatively identified as cricket iridovirus (CrIV) or insect iridovirus type 6 (IIV-6) [they are considered to be the same species of virus, with CrIV a variant of IIV-6 (Jakob et al., 2002)]. Primers for the major capsid protein of this virus produced a single amplicon from DNA extracted from the fat body. Sequencing of the PCR amplicon confirmed its identity as *Gryllus bimaculatus* iridovirus major capsid protein ( $E$  value=0.0). Cricket haemolymph was found to contain viral proteins, including the major capsid protein of IIV-6/CrIV.

### Viral effects on reproductive tissue in *G. texensis*

The spermathecae were full of sperm, demonstrating that infected females were sexually active ( $N=18/18$  infected females). The lateral oviducts typically had few or no eggs ( $N=18$  infected females; all females had fewer than 10 eggs per lateral oviduct). Normally, lateral oviducts hold ~120 eggs (Adamo and Baker, 2011). The ovaries contained few or no developing follicles.

In contrast, observations of the testes of infected male crickets suggested that there was no gross decrease in size, although

testicular size was not quantified ( $N=7$ ). The spermatophore of infected males held sperm (4/4 males). However, the sperm showed little or no motility ( $N=4$ ), with the sperm remaining inside, or close to, the remains of the crushed spermatophore during the 10 min observation period. In contrast, sperm liberated from the spermatophores of control males ( $N=3$ ) moved vigorously away from the remains of the spermatophore during the same time period.

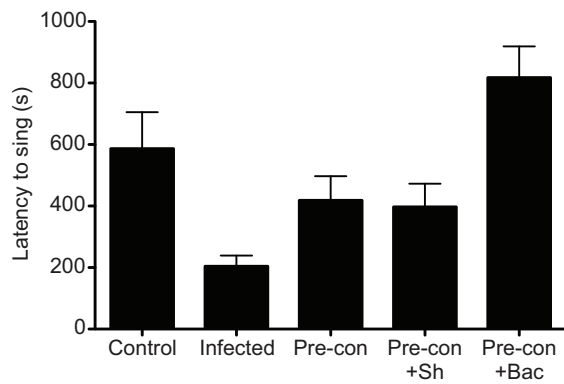
There was no difference in live mass between infected crickets ( $N=13$  males,  $N=18$  females) and uninfected ( $N=8$  males,  $N=10$  females) crickets (two-way ANOVA,  $F_{1,45}=0.06$ ,  $P=0.80$ ), although females ( $N=28$ ) were heavier than males ( $N=21$ ; two-way ANOVA,  $F_{1,45}=22.8$ ,  $P<0.0001$ ; no significant interaction,  $F_{1,45}=0.19$ ,  $P=0.67$ ). There was also no difference in size between infected and control crickets as estimated by femur length (two-way ANOVA,  $F_{1,45}=0.10$ ,  $P=0.38$ ; sex  $F_{1,45}=0.78$ ,  $P=0.38$ ; interaction  $F_{1,45}=0.77$ ,  $P=0.38$ ; sample sizes as shown above).

### Evidence for sexual transmission

None of the sentinel animals ( $N=4$ ) showed evidence of infection. The fat body appeared normal and was devoid of viral DNA. Of the six uninfected females courted by infected males, three became infected after physical contact with males during sexual behaviour, including one that did not copulate with an infected male, but was merely courted by that male (courting includes close physical contact). Of the four uninfected males that were placed with infected females, two became infected after mating with infected females. However, one male remained uninfected even after he copulated with an infected female.

### Behavioural effects of the virus

There was no significant difference in anti-predator behaviour between infected and uninfected crickets (Mann–Whitney,  $U=150$ ,  $P=0.5$ ; control  $N=12$ , infected  $N=29$ ). Examining locomotion



**Fig. 2. Infected males have a shorter latency to sing than controls.** The bars denote means, and the error bars represent s.e.m. Pre-con were uninfected crickets tested prior to the infection entering the colony. Pre-con+Sh were uninfected sham-challenged crickets tested prior to the infection entering the colony. Pre-con+Bac were uninfected, immune-challenged crickets tested prior to the infection entering the colony.

separately, we found no difference in locomotion between infected and uninfected crickets (Mann–Whitney,  $U=160$ ,  $P=0.70$ ; control  $N=12$ , infected  $N=29$ ). Infected and uninfected crickets did not differ significantly when compared with control, uninfected crickets from the same colony run 6 months previously [ $N=23$ ; Kruskal–Wallis  $H=0.57$ ,  $P=0.75$  (Adamo et al., 2013)].

#### Effect of infection on sexual behaviour

Infected male crickets were as likely to court females (43.8%,  $N=16$ ) during their initial presentation as were uninfected crickets [50%,  $N=6$ ;  $G$ -test (1 d.f.)=0.07,  $P=0.79$ ]. Taking the average value of three trials, infected males ( $N=7$ ) had a shorter latency to court (i.e. producing courtship song) than uninfected males ( $N=5$ , Mann–Whitney  $U=0.001$ ,  $P=0.0025$ , Fig. 2). However, uninfected males challenged with heat-killed *Serratia marcescens* ( $N=12$ ) took longer to court females compared with sham-injected ( $N=12$ ) or unchallenged controls ( $N=12$ ) ( $F_{2,33}=7.6$ ,  $P=0.002$ ; Tukey's multiple comparison, no significant difference between control and sham-injected crickets  $q=0.25$ ,  $q=4.6$ ,  $P>0.05$ ) (Fig. 2).

There was no significant difference in the time required for infected females ( $N=5$ ) to mount a courting male (Kruskal–Wallis=0.65,  $P=0.73$ ) compared with controls ( $N=8$ ).

#### Effect of infection on sickness behaviour (illness-induced anorexia)

There was no evidence of illness-induced anorexia in infected crickets ( $N=21$ ) even after injection of heat-killed bacteria ( $N=11$ ) (Fig. 3;  $F_{3,52}=0.12$ ,  $P=0.95$ , sham-injected crickets  $N=11$ , uninfected controls  $N=11$ ). Healthy crickets that were tested prior to the infection of the colony ( $N=16$ ) significantly reduced feeding when immune challenged with heat-killed *S. marcescens* ( $N=16$ ) (Fig. 3;  $t_{30}=3.96$ ,  $P=0.0004$ ).

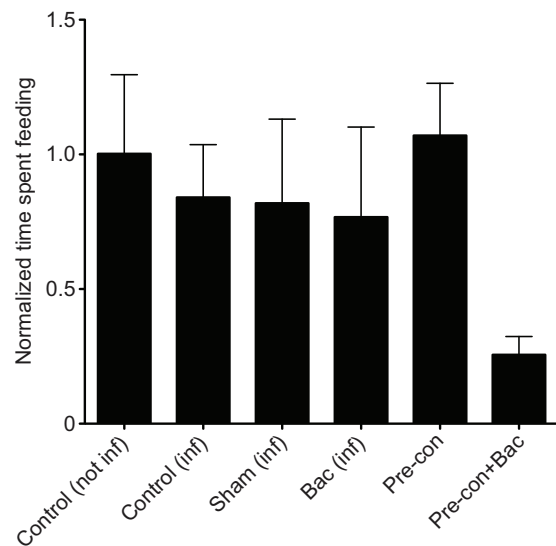
#### Physiological effects

##### Haemocyte count

Total haemocyte count did not differ between infected ( $N=7$ ) and uninfected ( $N=8$ ) crickets ( $t_{13}=1.13$ ,  $P=0.28$ ).

##### Total haemolymph protein and phenoloxidase activity

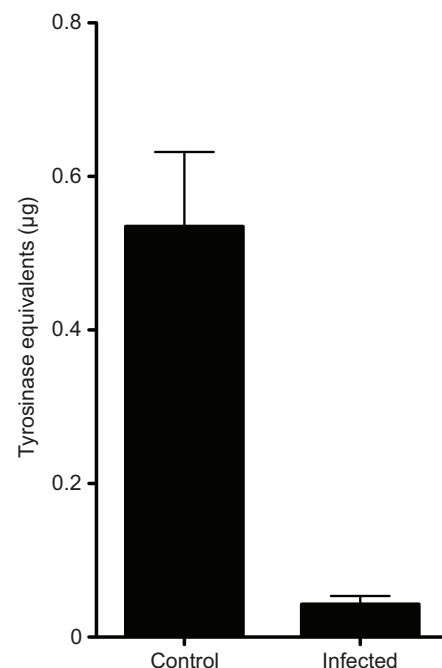
Infected crickets had significantly less total protein in their haemolymph ( $349.8\pm 12.02$  mg ml<sup>-1</sup>,  $N=46$ ) than did controls



**Fig. 3. Infected crickets show no evidence of illness-induced anorexia.**

Bars denote means and error bars denote s.e.m. All values were normalized against the average time uninfected crickets spent feeding (not inf, uninfected; inf, infected). Pre-con were uninfected crickets tested prior to the infection entering the colony. Pre-con+Bac were uninfected, immune-challenged crickets tested prior to the infection entering the colony.

( $570.5\pm 21.33$  mg ml<sup>-1</sup>,  $N=67$ ) ( $t_{111}=7.99$ ,  $P<0.0001$ ). We noted anecdotally that infected crickets took longer to melanize their wounds than controls. This observation led us to measure phenoloxidase activity, an enzyme important for melanization (Chapman, 1998). Total phenoloxidase activity was also significantly lower in infected crickets ( $N=55$ ) than in control crickets ( $N=55$ ;  $t_{108}=2.44$ ,  $P=0.016$ ; Fig. 4). Total phenoloxidase activity was significantly lower in infected crickets even when



**Fig. 4. Infected crickets have lower total phenoloxidase activity.** Bars denote means and error bars represent s.e.m.

corrected for total protein content in the blood ( $t_{86}=5.29$ ,  $P<0.0001$ ,  $N=58$  infected,  $N=30$  control). There was a significant correlation between total protein content and phenoloxidase activity in infected animals (Pearson's  $r^2=0.40$ ,  $P=0.001$ ,  $N=58$ ). Controls ( $N=20$ ) also had greater inducible *in vitro* phenoloxidase activity relative to baseline values than did infected crickets ( $N=12$ ; Mann–Whitney,  $U=28.5$ ,  $P=0.005$ ).

## DISCUSSION

Using both molecular methods and electron microscopy, we were able to identify the pathogen in our cricket colony as insect iridescent virus, type 6 (IIV-6, cricket variant CrIV). We also showed that IIV-6, like some other insect viruses (van Houte et al., 2013), is capable of altering host behaviour. Infected crickets began courtship singing sooner than controls (Fig. 2). Moreover, sickness behaviour was curtailed (Fig. 3). These two changes would tend to produce a similar result – a continuation of sexual behaviour in infected animals. However, host anti-predator behaviour, a behaviour critical for host survival (Adamo et al., 2013), was indistinguishable from that of controls. This new host behavioural phenotype is likely to enhance viral transmission.

Our results suggest that physical contact, a key feature of sexual behaviour, is capable of transmitting the virus (also see Hunter et al., 2003). Crickets contact each other frequently during courtship; for example, both males and females brush their antennae over each other's cuticle (Adamo and Hoy, 1994). Crickets groom their antennae with their mouthparts during courtship (Adamo and Hoy, 1994), and this could transfer particles from the antennae into the mouth. *Per os* infection is thought to be the main route of infection for this virus (Williams et al., 2005). Our sentinels remained healthy, with no evidence of viral DNA in their fat body, suggesting that merely being in the same room as infected animals is not sufficient for infection. This is consistent with other papers on IIV-6 and cricket hosts (e.g. Kleepsies et al., 1999). Other possible modes of viral transmission may be rare in this cricket. For example, infection from viral particles shed into the environment may be uncommon. Humidity levels are often low near Austin (TX, USA) (Ward, 2010), and these particles lose activity rapidly on dry soil (Williams et al., 2005). Cricket densities are usually low in the field (Cade and Cade, 1992), and this species is asocial. Males may be able to infect each other during aggressive behaviour (Williams et al., 2005), but this behaviour only occurs within the context of sexual behaviour (Loher and Dambach, 1989). Iridoviruses can also be spread by cannibalism (Williams and Hernandez, 2006). Crickets will cannibalize each other (Adamo et al., 2010), but this may be an uncommon event in the field because of competition from other scavengers for cricket corpses. We found no evidence of infection of the testes, supporting the hypothesis that this virus is not vertically transmitted (Williams et al., 2005). Furthermore, Just and Essbauer (Just and Essbauer, 2001) found no sign of infection in the ovaries and testes of a related cricket (*Gryllus bimaculatus*) infected with the same virus. Therefore, host sexual behaviour may be important for transmission under natural conditions.

Total protein concentration in the haemolymph was lower in infected animals than in controls. Most of the proteins in insect haemolymph are made by the fat body (Chapman, 1998). Therefore, this result is consistent with the hypothesis that the virus redirects the host fat body towards making viral particles and away from making host proteins. One of the most abundant proteins in the haemolymph of females is vitellogenin, a yolk protein made by the fat body (Chapman, 1998). The steep decline in total protein in the haemolymph suggests that the amount of vitellogenin available to

the ovaries is probably also greatly reduced during infection. This reduction could explain the loss of egg production; eggs cannot be made without the yolk protein. The testes may be less affected by infection because they rely less on proteins made by the fat body.

We do not know how the virus alters host behaviour, but our results provide some indirect evidence suggesting a mechanism. Whether the production of all host proteins was equally suppressed during infection is unknown, but total phenoloxidase activity (Fig. 4) was significantly reduced, suggesting that less prophenoloxidase is being made. Phenoloxidase is virucidal (Shelby and Popham, 2006); therefore, this decline would probably benefit the virus. The decline in the production of immune-related compounds by the fat body may also help explain the lack of sickness behaviour. In insects, cytokines (e.g. immune activators like ENF family peptides) are made by the fat body and/or haemocytes (Kamimura, 2012). Cytokines are responsible for inducing sickness behaviours in vertebrates (Dantzer et al., 2008), and are thought to be involved in inducing the same phenomena in invertebrates (Adamo, 2008). Viral infection can trigger cytokine release in insects, leading to the induction of an anti-viral state (Kemp and Imler, 2009). Preventing this state probably benefits the virus. If the virus inhibits the host's immune system from producing immune-related peptides and proteins such as cytokines, this could explain the decline in sickness behaviours in the host.

The ability of this virus to attack the fat body, a key producer of molecules important for immune function (Chapman, 1998), probably pre-adapts it for the ability to suppress the host's defence against it. However, even if the declines in sickness behaviour began as a side-effect of the virus's attack on the fat body, positive selection for these effects could lead to augmentation of these changes, with a diminution of effects on fat body proteins needed for host survival. More research is needed to determine whether there is positive selection for the virus to specifically suppress host sickness behaviour [see Poulin for a discussion of this issue (Poulin, 2010)].

The increase in male sexual behaviour, and the continuation of female sexual behaviour, is unlikely to be a host response, i.e. terminal reproductive investment. Terminal reproductive investment, an increase in reproduction prior to death, is known to occur in female crickets (Adamo, 1999; Shoemaker et al., 2006). However, because infected females have few eggs, continued mating is unlikely to increase their fitness, especially as they are capable of storing sperm from previous matings (Loher and Dambach, 1989). Infected males have low motility sperm and may be infertile. Therefore, it is unlikely that increased mating benefits males.

Because the infection of our colony was unintentional, the infected animals could represent a non-random subset of our colony. Such a subset might be different from control crickets regardless of any direct viral effects. However, infected animals often had assay values beyond the range of values found within our colony animals (e.g. total phenoloxidase activity, Fig. 4). These values are pathological and not likely to be the result of the virus selecting for an unusual subset of the population. Similarly, crickets in our control group were sometimes obtained from a different colony, although these animals were descended from crickets from our lab colony. However, we do not believe there were any significant differences between the two populations of crickets. For example, with sexual behaviour, we compared our infected colony animals with historical controls (data collected from animals prior to the infection of the colony) as well as with uninfected animals from Ottawa. The historical controls and the uninfected animals from Ottawa did not differ in their mating behaviour, suggesting that there were no significant differences between the two populations.

Sexually transmitted infections (STIs) are widespread in insects (Knell and Webberley, 2004; Burand et al., 2012) and other animals (Lockhart et al., 1996). STIs commonly result in reductions in fertility (Knell and Webberley, 2004); therefore, animals will be selected to avoid infected conspecifics. Nevertheless, there is no evidence that insects avoid mating with conspecifics infected with STIs, even though some insects (e.g. ants) avoid individuals infected with non-STIs (de Roode and Lefèvre, 2012). In fact, some animals infected with STIs are able to attract even more mating partners than uninfected controls [insects (Knell and Webberley, 2004; Goodacre and Martin, 2012), vertebrates (Dass et al., 2011)]. The popularity of mates infected with STIs is surprising as animals are typically very sensitive to the state of a prospective mate's health, and avoid mating with conspecifics with contagious diseases (Able, 1996). As Knell and Webberley point out, a host that behaves as if it were sick will not attract mates (Knell and Webberley, 2004). Suppression of sickness behaviour may be critical for the ability of infected individuals to secure mating partners – an ability that is key for the transmission of any STI (Adamo, 2014).

Sickness behaviour not only makes an individual less desirable as a mating partner but also reduces the mating effort of infected animals. For example, Jacot et al. (Jacot et al., 2004) found that immune activation reduces calling effort in male crickets (*Gryllus campestris*). A reduction in calling effort will reduce the number of mating partners the animal attracts (Gerhardt and Huber, 2002). Our results show that immune activation also decreases the ability of males to court any female that was attracted, further reducing the opportunity to contact another cricket. Mating rate is a key determinant of transmission for STIs (Webberley et al., 2006). Therefore, sickness behaviours would decrease transmission of an STI like IIV-6/CrIV. Suppression of other aspects of sickness behaviour, such as illness-induced anorexia, might also benefit an STI by preventing a decline in the availability of resources for pathogen reproduction. In mammals, STIs are more likely to be asymptomatic than other diseases (Lockhart et al., 1996; Mackey and Immerman, 2003; Antonovics et al., 2011), suggesting that the suppression of sickness behaviour may be widespread in STIs (Adamo, 2014).

## MATERIALS AND METHODS

### Animals

Crickets (long-winged *G. texensis*) were originally collected near Austin, TX, USA, and have been maintained as a laboratory colony for many generations. Pellets of dry cat food and water were provided *ad libitum* during rearing. Crickets were reared at 25±2°C on a 12 h/12 h light/dark cycle. All studies were approved by the Animal Care Committee of Dalhousie University (no. I11-025) and are in accordance with the Canadian Council on Animal Care. Adult crickets were used between 8 and 21 days after the moult to adulthood. At this age they are sexually mature (Cade and Wyatt, 1984; Solymar and Cade, 1990) but still within their lifespan in the field (Murray and Cade, 1995). Illness-induced anorexia studies were performed on last instar nymphs. All behavioural trials were conducted during mid-photophase.

Chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise noted.

### Source of infected animals

Some females in our colony stopped laying eggs. On dissection these females exhibited a hypertrophic fat body with an iridescent blue sheen. The colony was quarantined. Uninfected animals were obtained from Dr S. Bertram's colony at Carleton University in Ottawa, Canada. The animals from Dr Bertram's colony were descended from animals shipped from our colony to her laboratory about 2 years previously, and were therefore from the same genetic pool. The crickets from Dr Bertram's colony were

held under similar conditions to our infected colony but in a separate room to prevent inadvertent infection. These animals were tested using PCR to confirm their uninfected status.

### Identification of virus

#### Electron microscopy

Tissues to be processed for thin sectioning were dissected out into the primary fixative (3% glutaraldehyde/1% acrolein in 50 mmol l<sup>-1</sup> sodium cacodylate buffer containing 250 mmol l<sup>-1</sup> sucrose) and left at room temperature for 2 h. Post-fixation was in 2% OsO<sub>4</sub> in the same buffer, followed by overnight staining in 0.1% aqueous uranyl acetate. Embedment was in epon/araldite resin, following dehydration in acetone. Micrographs were taken using a Jeol JEM 1230 electron microscope operating at 80 kV, using a 12 HR digital Hamamatsu C4742-51 camera.

To determine whether any common bacterial or fungal pathogens were contributing to the infection, we aseptically harvested fat body and haemolymph and plated these on nutrient agar plates.

#### PCR for molecular identification of the virus

Fat body, spermatheca (females), spermatophore and testes (males) were dissected under sterile conditions. During the dissection, the colour of the fat body, number of eggs in the lateral oviducts, and whether the spermatheca contained sperm were determined. Spermatophores were opened using fine dissecting scissors in about 200 µl of phosphate-buffered saline (PBS) and examined using phase contrast microscopy. Samples were frozen at -80°C until use.

DNA was extracted from thawed samples using a DNAeasy kit (Qiagen) following the manufacturer's instructions. Approximately 300 ng of DNA was used as a template in a 30 µl PCR reaction, including 0.4 µmol l<sup>-1</sup> of each primer, 0.2 mmol l<sup>-1</sup> dNTPs, 1.5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 1×PCR buffer and 2 U *Taq* polymerase (Fermentas, <http://www.thermoscientificbio.com/fermentas/>). Cycling was performed under the following conditions: 5 min at 95°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, followed by final extension at 72°C for 10 min. The forward and reverse primer sequences of the major capsid protein gene were PCRFOR: 5'-CCATTACATTTAA-TGATTTGG-3' and PCRREV: 5'-TTTGTGACGTGGTGCAGTTTGAAC-3' (see Weinmann et al., 2007). PCR products were run on a 1.5% agarose gel in 0.5× Tris base/boric acid/EDTA buffer solution and stained with gel green following amplification with the Weinmann primers. PCR products were treated with ExoSap (Affymetrix, [www.affymetrix.com](http://www.affymetrix.com)) and a 510 bp amplicon was sent for sequencing. Mega 5 (<http://www.megasoftware.net/>) and Bioedit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) were used to edit sequence data and produce contiguous alignments. Homologous sequences were identified using BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with overlapping regions removed.

#### Identification of viral proteins in haemolymph

Viral proteins were identified through sodium dodecyl sulphate polyacrylamide gel electrophoresis. A 4 µl sample of haemolymph was collected under sterile conditions from crickets using a 10 µl automatic pipetter and an ice-cold pipetter tip flushed with anti-coagulant [0.15 mol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> EDTA, 10 mmol l<sup>-1</sup> glutathione and a few crystals of phenothiocarbamide and protease inhibitor cocktail (enough to form a supersaturated solution)]. The samples were placed in 12 µl of phosphate-buffered saline (PBS) and frozen at -80°C until use. Each sample was diluted 1:2 using loading buffer with dithiothreitol and phenylthiocarbamide. The tubes were left to incubate in boiling water for 3 min. The samples were run in Mini-PROTEAN TGX Precast Gels (Bio-Rad) at 150 V for 90 min. Proteins were visualized by silver staining (Swain and Ross, 1995). A strong band visible in the lanes of infected samples was sent out for sequencing (Proteomics Core Facility, Dalhousie University).

#### Evidence for sexual transmission

To test whether the virus could also be transmitted via sexual contact, we placed infected male and female crickets with uninfected crickets. Infected and uninfected crickets were isolated into single containers (17×15×9.5 cm) for at least 1 day prior to the mating trial. Both male and female crickets were transferred to a clean, fresh container (17×15×9.5 cm) separated by a

divider. Infected males were paired with uninfected females ( $N=6$ ), and infected females were paired with uninfected males ( $N=4$ ). After 1 min the divider was removed. The time to first contact was recorded, as was the time until the male began to sing (i.e. latency to singing). The time of each female mounting was also noted, as was the time of copulation, if any. We collected the fat body from crickets 10 days after the courtship trials described above. Kleespies et al. found that crickets began to die 2 weeks after infection (Kleespies et al., 1999). Four crickets (two male and two female) acted as sentinels. They were housed in the same room, but did not participate in mating trials with infected crickets. Their fat body was collected at the same time. The harvested fat body was tested for virus using PCR as described above.

## Behavioural effects

### Effects of infection on anti-predator behaviour

A modified plus maze was used to assess anti-predator behaviour (see Adamo et al., 2013). Briefly, the plus maze was shaped like a plus sign and constructed of black acrylic. It consisted of four arms ( $14 \times 8 \times 6.5$  cm each) and an open central area ( $8 \times 8 \times 6.5$  cm). During the trial, two opposing arms were covered with heavy black bristle board, creating dark spaces underneath. Crickets were gently transferred from their home cage to the central, uncovered area. They remained under the transfer cup for 1 min. The cup was then removed and we measured the time the cricket remained motionless. We called this time period 'freezing'. Freezing is a stereotypic anti-detection response associated with predation avoidance across taxa (Stynoski and Noble, 2012) including crickets (Niemelä et al., 2012). We also measured the number of times each cricket entered an arm of the plus maze as an assessment of its tendency to explore its environment. We measured the amount of time each cricket spent locomoting as a proxy for behavioural activity. We also measured the time it spent under covered arms to assess its tendency to avoid open spaces. The apparatus was cleaned with disinfectant between trials. The person scoring the trials was blind to the infection status of the cricket. There are no sex differences in plus maze performance (Adamo et al., 2013); therefore, data from both sexes were pooled.

### Effect of infection on sexual behaviour

We assessed the latency (time to singing) and duration of courtship behaviour in male and female infected and uninfected crickets. Infected and uninfected crickets were isolated into single containers ( $17 \times 15 \times 9.5$  cm) for at least 1 day prior to the mating trial. Both male and female crickets were transferred to a clean, fresh container ( $17 \times 15 \times 9.5$  cm) separated by a divider. After 1 min the divider was removed. The time to first contact was recorded, as was the time until the male began to sing. The time of each female mounting was also noted, as was the time of copulation, if any. If there was no mating after 40 min, crickets were re-tested with a fresh partner for a further 40 min.

We also tested whether an immune challenge affected the latency of healthy male crickets to produce courtship song. Male crickets were given a 2  $\mu$ l injection of heat-killed *S. marcescens* (approximately  $1 \times 10^4$  cells) in sterile water, or acted as an unhandled control. Ninety minutes later, males were given mating trials with uninfected females as described above. This time point was chosen because there is a clear physiological response (i.e. lipid release) 90 min after injection of heat-killed *S. marcescens* (Adamo et al., 2008).

### Effect of infection on sickness behaviour (illness-induced anorexia)

Anecdotal observations suggested that virus-infected crickets showed normal behaviour until close to death. We tested whether infected crickets could express illness-induced anorexia, a robust response to immune activation in crickets (Adamo et al., 2010). The experiment followed a previously used procedure (Adamo et al., 2010). Infected crickets were deprived of food, but not water, for 2 days. They were then given a 2  $\mu$ l injection of heat-killed *S. marcescens* (approximately  $1 \times 10^4$  cells) or a 2  $\mu$ l injection of sterile water, or acted as an unhandled control. Injections of heat-killed *S. marcescens* are known to induce illness-induced anorexia in this species (Adamo et al., 2010). One hour after injection, crickets were placed on one side of an experimental chamber ( $17 \times 15 \times 9.5$  cm) with a food pellet in the other, and the amount of time they spent feeding over the

30 min trial was recorded. These times were chosen based on the earlier study (Adamo et al., 2010). Adamo et al. found no effect of sex on illness-induced anorexia, so information on the two sexes was pooled (Adamo et al., 2010). The results were also compared with data collected on uninfected animals.

## Physiological effects – evidence for alteration of immune function

### Haemocyte count

Haemocyte count was obtained by removing 4  $\mu$ l of haemolymph from crickets using a 10  $\mu$ l automatic pipette and an ice-cold pipette tip flushed with anti-coagulant [ $0.15 \text{ mol l}^{-1}$  NaCl,  $10 \text{ mmol l}^{-1}$  EDTA,  $10 \text{ mmol l}^{-1}$  glutathione and a few crystals of phenothiocarbamide and protease inhibitor cocktail (enough to form a supersaturated solution)]. Haemolymph was transferred to 200  $\mu$ l of ice-cold anti-coagulant and gently mixed, and then a 10  $\mu$ l sample was placed on a Fuchs–Rosenthal counting chamber within 1 min of collection. Cells were counted using phase-contrast microscopy. Haemocyte number does not differ between the sexes (Adamo, 2010); therefore, results from males and females were pooled.

### Total haemolymph protein and phenoloxidase activity

To measure the total protein content of the haemolymph, 4  $\mu$ l of haemolymph was removed as described above from both infected and uninfected crickets. The haemolymph was added to 60  $\mu$ l of ice-cold phosphate-buffered saline and immediately vortexed for 5 s. A 10  $\mu$ l sample of the haemolymph/saline mixture was then added to 180  $\mu$ l of Bradford reagent in a 96-well plate. After a 15 min incubation with the reagent, the absorbance was read at 590 nm. Each assay was run with standards of bovine serum albumen.

Phenoloxidase activity was measured using a method modified from Bidochka et al. (Bidochka et al., 1989). A 4  $\mu$ l sample of haemolymph was removed as described above from both infected and uninfected crickets. A 15  $\mu$ l sample of the haemolymph/saline mixture (see above) was transferred to a tube containing 20  $\mu$ l of water. Cricket haemolymph rarely exhibits any spontaneous activity (Adamo, 2004) and this tube provides a baseline measure. A further 15  $\mu$ l of the haemolymph/saline mixture was added to a tube containing 20  $\mu$ l of bovine pancreas alpha chymotrypsin ( $1.2 \text{ mg ml}^{-1}$ ) dissolved in water. The chymotrypsin converts prophenoloxidase into the active form (Adamo, 2004). This tube provides an estimate of the maximal potential activity. Another 15  $\mu$ l of the haemolymph/saline mixture was added to 18  $\mu$ l of water and 2  $\mu$ l of heat-killed *S. marcescens*. This tube assesses the amount of activation of prophenoloxidase during an immune challenge *in vitro*. The tubes were left to incubate at room temperature for 20 min. After the incubation period, tubes were centrifuged at room temperature at 10,000 for 5 min. A 30  $\mu$ l sample of supernatant was added to 180  $\mu$ l of  $0.02 \text{ mol l}^{-1}$  L-Dopa in a 96-well plate. After mixing, the change in absorbance at 490 nm was monitored for 20 min. Preliminary studies showed that this was within the linear range of this assay. Standards using mushroom tyrosinase were run as well on the same plate.

## Statistics

Statistics were performed using IBM SPSS Statistics (v. 19) and GraphPad Prism (v. 5.0b, GraphPad Software, La Jolla, CA, USA). A principal components analysis (PCA) was performed on the plus maze behavioural measures (locomotion, time spent freezing, the number of arm entries and the time spent in the covered arms) as described previously (Adamo et al., 2013). Only the first component of the PCA had an eigenvalue above 1 (score=2.1) and it explained 70% of the total variance ( $N=23$  crickets). The validity of the PCA was shown by the measure of sampling adequacy (Kaiser–Meyer–Olkin=0.46) and a highly significant Bartlett's test of sphericity ( $P<0.0001$ ). The first component of the PCA was used as a summary anti-predator score. Data were tested for normality; normally distributed data were tested with parametric statistics. Non-normal data were analysed using non-parametric statistics (Meddis, 1984). Non-parametric tests were also used for analyses in which at least one of the treatment groups had a sample size less than 6; Meddis argue that sample sizes smaller than this cannot be adequately tested for a normal distribution (Meddis, 1984).

**Acknowledgements**

We thank J. Beach, K. Bradley, E. Fairn and N. Parsons for assistance with behavioural trials. We thank A. Cohen for assistance with protein identification using LC-MS/MS.

**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

S.A.A. generated the hypothesis, designed the study, analyzed the data and wrote the paper. I.K. helped design and perform the behavioural assays, performed the PCR analysis, and critically read the paper. R.H.E. designed and helped perform the PCR analysis and critically read the paper. D.S. performed and analyzed the EM study and helped edit the paper.

**Funding**

This study was funded by NSERC (Natural Sciences and Engineering Research Council of Canada) grants to S.A.A. and D.S.

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