1   A viral aphrodisiac in the cricket *Gryllus texensis*

2

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5   transmitted infection

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29   We identified the insect iridovirus IIV-6/CrIV as a pathogen of the cricket *Gryllus*

30   *texensis* using electron microscopy (EM) and polymerase chain reaction (PCR) analysis.

31   Electron microscopy (EM) showed that the virus attacks the fat body, an organ important

32   for protein production, immune function and lipid storage. During infection the fat body

33   hypertrophied, but egg production withered, leaving the lateral oviducts empty of eggs;

34   the females were effectively sterile. EM of the testis of infected males suggests that the

35   testis was not invaded by the virus, although sperm taken from the spermatophores of

36   infected males showed little or no motility. Nevertheless, males and females continued to

37   mate when infected. In fact, infected males were quicker to court females than

38   uninfected controls. The virus benefits from the continued sexual behaviour of its host;

39   transmission studies show that the virus can be spread through sexual contact. Sickness

40   behaviour, the adaptive reduction of feeding and sexual behaviour that is induced by an

41   activated immune system, is absent in infected crickets. Total hemolymph protein is

42   reduced, as is phenoloxidase activity, suggesting a reduction in immune protein

43   production by the fat body. The evidence suggests that during IIV-6/CrIV infection, the

44   immune signal(s) that induce sickness behaviour are absent. Curtailment of a host's

45   sickness behaviour may be necessary for any pathogen that is spread by host sexual

46   behaviour.

47

48   List of Abbreviations

49

50   EDTA - ethylenediaminetetraacetic acid

51   EM - electron microscopy

52   ENF - Family of insect cytokines (consensus amino-terminal sequence Glu-Asn-Phe)

53   IIV-6 - insect iridovirus type 6

54   CrIV - Cricket iridovirus

55   PCR - polymerase chain reaction

56   STI - sexually transmitted infection

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62   Introduction

63

64   Viruses are known manipulators of host behaviour (Van Houte et al., 2013). For some

65   viruses, certain changes in host behaviour appear to be an important element in successful

66   viral transmission (e.g. Burand et al., 2012). In these systems, there will be intense

67   selection pressure on the virus to manipulate its host. The existence of this evolutionary

68   pressure provides us with the opportunity to discover how evolution has solved the

69   neurobiological problem of behavioural control. This problem is especially interesting in

70   the context of viruses as they tend to have small genomes, and so have a limited number

71   of potential molecular tools.

72

73   How viruses, or any other pathogen/parasite, alter host behaviour remains poorly

74   understood (Adamo, 2013; van Houte et al., 2013; Adamo, 2012; Lefèvre et al., 2009).

75   Most pathogens appear to use multiple mechanisms simultaneously to alter host

76   behaviour, as opposed to relying on a 'magic bullet' approach (Adamo, 2013). One

77   mechanism that appears to be a common route leading to altered host behaviour is the

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

79

80   Signaling molecules produced by the immune system (e.g. cytokines) can induce and/or

81   suppress a wide range of host behaviours in both vertebrates (Dantzer, 2008) and

82   invertebrates (Adamo, 2008). These immune-induced changes in behaviour are known

83   collectively as 'sickness behaviours', and they help animals recover from infection (Hart,

84   1988). Such immune/neural connections may be especially prone to disruption by

85   parasitic manipulators, because all parasites are under heavy selection to interfere with

86   host immune function (e.g. by altering cytokine production). Hijacking immune/neural

87   communication pathways may be a small evolutionary step (Adamo, 2013). In some

88   host/pathogen systems it may be important to prevent host behavioural responses to

89   infection (i.e. sickness behaviours) as these behaviours may reduce viral transmission

90   and/or interfere with viral manipulation of behaviour.

91

92   Some viruses are transmitted by sexual contact (e.g. Burand et al., 2012). Some of these

93   viruses cause host sterility; for example, the *Helicovera zea* nudivirus 2 sterilizes its moth

94   host, *Helicoverpa zea* (Burand et al., 2004). Therefore, continued sexual behaviour does

95   not increase host fitness. It is, however, vital for viral transmission (Burand et al., 2004).

96   Females infected with *Helicovera zea* nudivirus 2 show increased sexual behaviour,

97   leading to enhanced viral transmission (Burand et al., 2005). Although the mechanistic

98   details remain to be determined, the virus appears to use multiple methods to manipulate

99   host sexual behaviour (Burand et al., 2005; Burand and Tan, 2006). One mechanism not

100 yet explored in this system is the effect of the virus on host sickness behaviour. In most

101 animals, an immune challenge results in a reduction in sexual behaviour (e.g. crickets,

102 Jacot et al., 2004). Therefore, all sexually transmitted pathogens probably need to

103 suppress the expression of host sickness behaviour in order to maintain or increase sexual

104 behaviour in their host.

105

106   In this paper we examine the effect of an iridovirus on the cricket *Gryllus texensis* (Cade

107 and Otte, 2000). Iridoviruses are large icosahedral, cytoplasmic DNA viruses that attack

108 invertebrates, reptiles and amphibians (Williams et al., 2005). The viruses are probably

109 common in field populations, and can cause epizootics (Williams et al., 2005). One type

110 of iridovirus (IIV-6; cricket iridovirus isolate (CrIV), Jakob et al., 2002) attacks crickets.

111 The fat body is the primary target (Just and Essbauer, 2001). It takes on an iridescent blue

112 sheen, due to the large number of viral particles that are arranged in a paracrystalline

113 array (Williams et al., 2005). The fat body is the major site of protein production in

114 insects, as well as a storage organ for lipid (Chapman, 1998). It is also critical for disease

115 resistance because it produces proteins vital for immune function (Chapman, 1998). The

116 hemocytes, the immune cells of the blood, are also infected (Kleepsies et al., 1999); in

117 other words, this virus invades all major immune tissues of the cricket. Therefore, this

118 virus is well placed to alter immune/neural communication in its host. For example, a

119 decrease in the production of host immune proteins could suppress the expression of the

120 host's sickness behaviour.

121

122 Results

123

124   Identity of the Virus

125

126   The blue sheen of the fat body suggested the presence of an iridovirus (Williams et al.,

127   2005). EM confirmed the presence of a large (approximately 150 nm) icosahedral virus

128   (Fig. 1). The fat body cells were heavily packed with virions. Viral particles were also

129   found in the tracheal epithelial cells and muscle. In some cells, virions formed small

130   paracrystalline arrays. Viral particles were not found in the cells of the testis.

131

132   No other pathogens were found. Culturing of fat body or hemolymph from infected

133   crickets (n=6 females and 4 males) produced no growth on nutrient agar plates,

134   suggesting that there were no common bacterial or fungal pathogens present.

135

136   Given the EM results, and from descriptions in the literature (e.g. Kleepies et al., 1999;

137   Williams et al., 2005), the virus was tentatively identified as cricket iridovirus (CrIV) or

138   insect iridovirus type 6 (IIV-6) (both are considered the same species of virus, with CrIV

139   a variant of IIV-6, Jakob et al., 2002). Primers for the major capsid protein of this virus

140   produced a single amplicon from DNA extracted from fat body. Sequencing of the PCR

141   amplicon confirmed its identity as *Gryllus bimaculatus* iridovirus major capsid protein (E

142   Value=0.0).

143

144   Cricket hemolymph was found to contain viral proteins, including the major capsid

145   protein of IIV-6/CrIV.

146

147   Viral effects on reproductive tissue in *Gryllus texensis*

148

149   The spermethecae were full of sperm, demonstrating that infected females were sexually

150   active (n=18/18 infected females). The lateral oviducts typically had few or no eggs

151   (n=18 infected females; all females had fewer than 10 eggs/lateral oviduct). Normally

152   lateral oviducts hold approximately 120 eggs (Adamo and Baker, 2011). The ovaries

153   contained few or no developing follicles.

154

155   In contrast, observations of the testes of infected male crickets suggested that there was

156   no gross decrease in size, although testicular size was not quantified (n=7). The

157   spermatophore of infected males held sperm (4/4 males). However, the sperm showed

158   little or no motility (n=4), with the sperm remaining inside, or close to, the remains of the

159   crushed spermatophore during the 10 min observation period. In contrast, sperm

160   liberated from the spermatophores of control males (n=3) moved vigorously away from

161   the remains of the spermatophore during the same time period.

162

163   There was no difference in live weight between infected crickets (n=13 males, 18

164   females) and uninfected (n=8 males, 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

166   ANOVA, $F_{(1, 45)}=22.8$, $p<0.0001$; no significant interaction, $F_{(1, 45)}= 0.19$, $p=0.67$).

167   There was also no difference in size between infected and control crickets as estimated by

168   femur length (Two-way ANOVA, $F_{(1, 45)}=0.10$, $p=0.38$; sex $F_{(1, 45)}=0.78$, $p=0.38$;

169   interaction $F_{(1, 45)}=0.77$, $p=0.38$; sample sizes as shown above).

170

171   Evidence for sexual transmission

172

173   None of the sentinel animals (n=4) showed evidence of infection. The fat body appeared

174   normal and was devoid of viral DNA. Of the 6 uninfected females courted by infected

175   males, 3 became infected after physical contact with males during sexual behaviour,

176   including one that did not copulate with an infected male, but was merely courted by that

177   male (courting includes close physical contact). Of the 4 uninfected males that were

178   placed with infected females, 2 became infected after mating with infected females.

179   However, one male remained uninfected even after he copulated with an infected female.

180

181   Behavioural effects of the virus.

182

183   There was no significant difference in anti-predator behaviour between infected and

184   uninfected crickets (Mann-Whitney, U=150, p=0.5; control n=12, infected n=29).

185   Examining locomotion separately, we found no difference in locomotion between

186   infected and uninfected crickets (Mann-Whitney, U=160, p=0.70; control n=12, infected

187   n=29). Infected and uninfected crickets did not differ significantly when compared to

188   control, uninfected crickets from the same colony run 6 months previously (n=23; Adamo

189   et al., 2013; Kruskal-Wallis *H*=0.57, p=0.75).

190

191   Effect of infection on sexual behaviour

192   Infected male crickets were as likely to court females (43.8% n=16) during their initial

193   presentation as were uninfected crickets (50%, n=6; G-test (1 df) = 0.07, p=0.79). Taking

194   the average value of 3 trials, infected males (n=7) had a shorter latency to court (i.e.

195   producing courtship song) than uninfected males (n=5, Mann-Whitney U=0.001,

196   p=0.0025, Fig. 2).   However, uninfected males challenged with heat-killed *S. marcescens*

197   (n=12) took longer to court females compared with sham injected (n=12) or unchallenged

198   controls (n=12) (F(2,33)=7.6, p=0.002. Tukey's multiple comparison, no significant

199   difference between control and sham-injected crickets q=0.25, q=4.6, p>0.05. Fig. 2).

200

201   There was no significant difference in the time required for infected females (n=5) to

202   mount a courting male (Kruskall-Wallis=0.65, p=0.73) compared with controls (n=8).

203

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

205

206   There was no evidence of illness-induced anorexia in infected crickets (n=21) even after

207   injection of heat-killed bacteria (n=11) (Fig. 3; F(3, 52)=0.12, p=0.95, sham-injected

208   crickets=11, uninfected controls, n=11).   Healthy crickets that were tested prior to the

209   infection of the colony (n=16) significantly reduced feeding when immune-challenged

210   with heat-killed *S. marcescens* (n=16) (Fig. 3; t(30)=3.96, p=0.0004).

211

212   Physiological Effects

213

214   Hemocyte Counts

215

216   Total hemocyte counts did not differ between infected (n=7) and uninfected (n=8)

217   crickets (t(13)=1.13, p=0.28).

218

219   Total Hemolymph Protein and Phenoloxidase Activity

220

221   Infected crickets had significantly less total protein in their hemolymph (349.8 ± 12.02

222   mg/mL, N=46) than did controls (570.5 mg/mL ± 21.33, N=67) (t(111)=7.99, p<0.0001).

223   We noted anecdotally that infected crickets took longer to melanize their wounds than

224   controls. This observation led us to measure phenoloxidase activity, an enzyme

225   important for melanization (Chapman, 1998). Total phenoloxidase activity was also

226   significantly lower in infected crickets (n=55) than in control crickets (n=55; t(108)=2.44,

227   p=0.016; Fig. 4). Total phenoloxidase activity was significantly lower in infected

228   crickets even when corrected for total protein content in the blood (t(86)=5.29, p<0.0001,

229   n=58 infected, n=30 control). There was a significant correlation between total protein

230   content and phenoloxidase activity in infected animals (Pearson's r^2=0.40, p=0.001,

231   n=58). Controls (n=20) also had greater inducible in vitro phenoloxidase activity relative

232   to baseline values than did infected crickets (n=12; Mann Whitney U=28.5, p=0.005).

233

234   Discussion

235

236   Using both molecular methods and electron microscopy, we were able to identify the

237   pathogen in our cricket colony as Insect Iridescent Virus, Type 6 (IIV-6, cricket variant

238   CrIV). We also showed that IIV-6, like some other insect viruses (Van Houte et al.,

239   2013), is capable of altering host behaviour. Infected crickets began courtship singing

240   sooner than controls (Fig. 2). Moreover, sickness behaviour was curtailed (Fig. 3). Both

241   of these changes would tend to produce a similar result – a continuation of sexual

242   behaviour in infected animals. However, host anti-predator behaviour, a behaviour

243   critical for host survival (Adamo et al., 2013), was indistinguishable from that of controls.

244   This new host behavioural phenotype is likely to enhance viral transmission.

245

246   Our results suggest that physical contact, a key feature of sexual behaviour, is capable of

247   transmitting the virus (also see Hunter et al., 2003). Crickets contact each other

248   frequently during courtship; for example, both males and females brush their antennae

249   over each other's cuticle (Adamo and Hoy, 1994). Crickets groom their antennae with

250   their mouthparts during courtship (Adamo and Hoy, 1994), and this could transfer

251   particles from the antennae into the mouth. *Per os* infection is thought to be the main

252   route of infection for this virus (Williams et al., 2005). Our sentinels remained healthy,

253   with no evidence of viral DNA in their fat body, suggesting that merely being in the same

254   room as infected animals is not sufficient for infection. This is consistent with other

255   papers on IIV-6 and cricket hosts (e.g. Kleepsies et al., 1999). Other possible modes of

256   viral transmission may be rare in this cricket. For example, infection from viral particles

257   shed into the environment may be uncommon. Humidity levels are often low near Austin,

258   Texas (Ward, 2010), and these particles lose activity rapidly on dry soil (Williams et al.,

259   2005). Cricket densities are usually low in the field (Cade and Cade, 1992), and this

260   species is asocial. Males may be able to infect each other during aggressive behaviour

261   (Williams et al., 2005), but this behaviour only occurs within the context of sexual

262   behaviour (Loher and Dambach, 1989). Iridoviruses can also be spread by cannibalism

263   (Williams et al., 2006). Crickets will cannibalize each other (Adamo et al., 2010), but

264   this may be an uncommon event in the field due to competition from other scavengers for

265   cricket corpses. We found no evidence of infection of the testes, supporting the

266   hypothesis that this virus is not vertically transmitted (Williams et al., 2005).

267   Furthermore, Just and Essbauer (2001) found no sign of infection in the ovaries and testes

268   of a related cricket (*Gryllus bimaculatus*) infected with the same virus. Therefore, host

269   sexual behaviour may be important for transmission under natural conditions.

270

271   Total protein concentration in the hemolymph was lower in infected animals than in

272   controls. Most of the proteins in insect hemolymph are made by the fat body (Chapman,

273   1998). Therefore, this result is consistent with the hypothesis that the virus redirects host

274   fat body towards making viral particles and away from making host proteins. One of the

275   most abundant proteins in the hemolymph of females is vitellogenin, a yolk protein made

276   by the fat body (Chapman, 1998). The steep decline in total protein in the hemolymph

277   suggests that the amount of vitellogenin available to the ovaries is probably also greatly

278   reduced during infection. This reduction could explain the loss of egg production; eggs

279 cannot be made without the yolk protein. The testes may be less affected by infection

280 because they rely less on proteins made by the fat body.

281

282 We do not know how the virus alters host behaviour, but our results provide some

283 indirect evidence suggesting a mechanism. Whether the production of all host proteins

284 was equally suppressed during infection is unknown, but total phenoloxidase activity

285 (Fig. 4) was significantly reduced, suggesting that less prophenoloxidase is being made.

286 Phenoloxidase is virucidal (Shelby and Popham 2006); therefore this decline would

287 probably benefit the virus. The decline in the production of immune-related compounds

288 by the fat body may also help explain the lack of sickness behaviour. In insects,

289 cytokines (e.g. immune activators like ENF family peptides) are made by the fat body

290 and/or hemocytes (Kamimura, 2012). Cytokines are responsible for inducing sickness

291 behaviours in vertebrates (Dantzer et al., 2008), and are thought to be involved in

292 inducing the same phenomena in invertebrates (Adamo 2008). Viral infection can trigger

293 cytokine release in insects, leading to the induction of an anti-viral state (Kemp and

294 Imler, 2009). Preventing this state probably benefits the virus. If the virus inhibits the

295 host's immune system from producing immune-related peptides and proteins such as

296 cytokines, this could explain the decline in sickness behaviours in the host.

297

298 The ability of this virus to attack the fat body, a key producer of molecules important for

299 immune function (Chapman, 1998), probably pre-adapts it for the ability to suppress the

300 host's defense against it. However, even if the declines in sickness behaviour began as a

301 side-effect of the virus's attack on the fat body, positive selection for these effects could

302 lead to augmentation of these changes, with a diminution of effects on fat body proteins

303 needed for host survival. More research is needed to determine whether there is positive

304 selection for the virus to specifically suppress host sickness behaviour (see Poulin, 2010

305 for a discussion of this issue).

306

307 The increase in male sexual behaviour, and the continuation of female sexual behaviour,

308 is unlikely to be a host response, i.e. terminal reproductive investment. Terminal

309 reproductive investment, an increase in reproduction prior to death, is known to occur in

310   female crickets (Adamo, 1999; Shoemaker et al., 2006). However, because infected

311   females have few eggs, continued mating is unlikely to increase their fitness, especially

312   as they are capable of storing sperm from previous matings (Loher and Dambach, 1989).

313   Infected males have low motility sperm and may be infertile. Therefore, it is unlikely

314   that increased mating benefits males.

315

316   Because the infection of our colony was unintentional, the infected animals could

317   represent a non-random subset of our colony. Such a subset might be different from

318   control crickets regardless of any direct viral effects. However, infected animals often

319   had assay values beyond the range of values found within our colony animals (e.g. total

320   phenoloxidase activity, Fig. 4). These values are pathological and not likely to be the

321   result of the virus selecting for an unusual subset of the population. Similarly, crickets in

322   our control group were sometimes crickets from a different colony, although these

323   animals were descended from crickets from our lab colony. However, we do not believe

324   there were any significant differences between the two populations of crickets. For

325   example, with sexual behaviour, we compared our infected colony animals with historical

326   controls (data collected from animals prior to the infection of the colony) as well as with

327   uninfected animals from Ottawa. The historical controls and the uninfected animals from

328   Ottawa did not differ in their mating behaviour, suggesting that there were no significant

329   differences between the two populations.

330

331   Sexually transmitted infections (STIs) are widespread in insects (Knell and Webberley,

332   2004; Burand et al., 2012) and other animals (Lockhart et al., 1996). STIs commonly

333   result in reductions in fertility (Knell and Webberley, 2004); therefore, animals will be

334   selected to avoid infected conspecifics. Nevertheless, there is no evidence that insects

335   avoid mating with conspecifics infected with STIs, even though some insects (e.g. ants)

336   avoid individuals infected with non-STIs (De Roode and Lefèvre, 2012). In fact, some

337   animals infected with STIs are able to attract even more mating partners than uninfected

338   controls (insects: Knell and Webberley, 2004; Goodacre and Martin, 2012; vertebrates:

339   Dass et al., 2011). The popularity of mates infected with STIs is surprising as animals are

340   typically very sensitive to the state of a prospective mate's health, and avoid mating with

341 conspecifics with contagious diseases (Able, 1996). As Knell and Webberley (2004)

342 point out, a host that behaves as if it were sick will not attract mates. Suppression of

343 sickness behaviour may be critical for the ability of infected individuals to secure mating

344 partners - an ability that is key for the transmission of any STI (Adamo, in review).

345

346 Sickness behaviour not only makes an individual less desirable as a mating partner, it

347 reduces the mating effort of infected animals. For example, Jacot et al., (2004) found that

348 immune activation reduces calling effort in male crickets (*Gryllus campestris*). A

349 reduction in calling effort will reduce the number of mating partners it attracts (Gerhardt

350 and Huber, 2002). Our results show that immune activation also decreases the ability of

351 males to court any female that was attracted, further reducing the opportunity to contact

352 another cricket. Mating rate is a key determinant of transmission for STIs (Webberley et

353 al., 2006). Therefore, sickness behaviours would decrease transmission of an STI like

354 IIV-6/CrIV. Suppression of other aspects of sickness behaviour, such as illness-induced

355 anorexia, might also benefit an STI by preventing a decline in the availability of

356 resources for pathogen reproduction. In mammals, STIs are more likely to be

357 asymptomatic than other diseases (Lockhart et al., 1996; Mackey and Immerman, 2003;

358 Antonovics et al., 2011), suggesting that the suppression of sickness behaviour may be

359 widespread in STIs (Adamo, in review).

360

361 Materials and Methods

362

363 Animals

364   Crickets (long-winged *G. texensis*) were originally collected near Austin, Texas

365 and have been maintained as a laboratory colony for many generations. Pellets of dry cat

366 food and water were provided ad lib during rearing. Crickets were reared at 25 +/- 2°C on

367 a 12/12h L/D cycle. All studies were approved by the Animal Care Committee of

368 Dalhousie University (#I11-025) and are in accordance with the Canadian Council on

369 Animal Care. Adult crickets were used between 8 and 21 days after the molt to

370 adulthood. At this age they are sexually mature (Cade and Wyatt, 1984; Solymar and

371 Cade, 1990) but still within their lifespan in the field (Murray and Cade, 1995). Illness-

372 induced anorexia studies were performed on last instar nymphs. All behavioural trials

373 were conducted during mid-photophase.

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

375 otherwise noted.

376

377 Source of infected animals

378   Some females in our colony stopped laying eggs. On dissection these females

379 exhibited a hypertrophic fat body with an iridescent blue sheen. The colony was

380 quarantined. Uninfected animals were obtained from Dr. S. Bertram's colony at Carleton

381 University in Ottawa, Canada. The animals from Dr. Bertram's colony were descended

382 from animals shipped from our colony to her laboratory about two years previously, and,

383 were therefore were from the same genetic pool. The crickets from Dr. Bertram's colony

384 were held under similar conditions to our infected colony, but in a separate room to

385 prevent inadvertent infection. These animals were tested using PCR to confirm their

386 uninfected status.

387

388 Identification of Virus

389 Electron Microscopy

390 Tissues to be processed for thin sectioning were dissected out into the primary fixative

391 (3% glutaraldehyde/1% acrolein in 50mM sodium cacodylate buffer containing 250mM

392 sucrose) and left at room temperature for 2 hrs. Postfixation was in 2% OsO_4 in the same

393 buffer, followed by overnight staining in 0.1% aqueous uranyl acetate. Embedment was

394 in epon/araldite resin, following dehydration in acetone. Micrographs were taken using a

395 Jeol JEM 1230 electron microscope operating at 80 kV, using a 12 HR digital

396 Hamamatsu C4742-51camera.

397

398 To determine whether any common bacterial or fungal pathogens were contributing to the

399 infection, we aseptically harvested fat body and hemolymph and plated these on nutrient

400 agar plates.

401

402 PCR for molecular identification of the virus

14

403 Fat body, spermetheca (females), spermatophore and testes (males) were dissected under

404 sterile conditions. During the dissection, the colour of the fat body, number of eggs in the

405 lateral oviducts, and whether the spermetheca contained sperm were determined.

406 Spermatophores were opened using fine dissecting scissors in about 200 µL of phosphate

407 buffered saline (PBS) and examined using phase contrast microscopy. Samples were

408 frozen at -80 C until use.

409

410 DNA was extracted from thawed samples using a DNAeasy kit (Qiagen) following the

411 manufacturer's instructions. Approximately 300 ng of DNA was used as a template in a

412 30 µl PCR reaction, including 0.4 µM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl2,

413 1×PCR buffer and 2U *Taq* polymerase (Fermentas, http://www.fermentas.com). Cycling

414 was performed under the following conditions: 5 min at 95ºC, 30 cycles of 1 min at 94ºC,

415 1 min at 50ºC, and 2 min at 72ºC, followed by final extension at 72ºC for 10 min. The

416 forward and reverse primer sequences of the major capsid protein gene were PCRFOR:

417 5'-CCATTACATTTAATGATTTGG-3' and PCRREV: 5'-

418 TTTTGACGTGGTGCAGTTTGAAC-3' and were taken from (Weinmann et al., 2007).

419 PCR products were run on a 1.5% agarose gel in 0.5X Tris base/boric acid/EDTA buffer

420 solution and stained with gel green following amplification with the Weinmann primers.

421 PCR products were treated with ExoSap (Affymetrix, USBtechsupport@affymetrix.com)

422 and a 510 bp amplicon was sent for sequencing. Mega 5 (http://www.megasoftware.net/)

423 and Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) were used to edit sequence

424 data and produce contiguous alignments. Homologous sequences were identified using

425 BLAST searches (http://blast.ncbi.nlm.nih.gov/) with overlapping regions removed.

426

427 Identification of Viral Proteins in Hemolymph

428 Viral proteins were identified though sodium dodecyl sulfate polyacrylamide gel

429 electrophoresis. 4 µL of hemolymph was collected under sterile conditions from crickets

430 using a 10 µL automatic pipetter and an ice-cold pipetter tip flushed with anti-coagulant

431 (0.15 M NaCl, 10 mM EDTA, 10 mM glutathione and a few crystals of

432 phenothiocarbamide and protease inhibitor cocktail (enough to form a supersaturated

433 solution)). The samples were placed in 12 µL of phosphate buffer saline and frozen at -

15

434 80C until use. Each sample was diluted 1:2 using loading buffer with dithiothreitol and

435 phenylthiocarbamide. The tubes were left to incubate in boiling water for 3 min. The

436 samples were run in Mini-PROTEAN TGX Precast Gels (BioRad) at 150 V for 90 min.

437 Proteins were visualized by sliver staining (Swain and Ross, 2005). A strong band visible

438 in the lanes of infected samples was sent out for sequencing (Proteomics Core Facility,

439 Dalhousie University).

440

441 Evidence for Sexual Transmission

442

443 To test whether the virus could also be transmitted via sexual contact, we placed infected

444 male and female crickets with uninfected crickets. Infected and uninfected crickets were

445 isolated into single containers (17cm x 15cm x 9.5cm) for at least one day prior to the

446 mating trial. Both male and female crickets were transferred to a clean, fresh container

447 (17cm x 15cm x 9.5cm) separated by a divider. Infected males were paired with

448 uninfected females (n=6), and infected females were paired with uninfected males (n=4).

449 After 1 min the divider was removed. The time to first contact was recorded, as was the

450 time until the male began to sing (i.e. latency to singing). The time of each female

451 mounting was also noted, as was the time of copulation, if any. We collected the fat body

452 from crickets 10 days after the courtship trials described above. Kleepsies et al., (1999)

453 found that crickets began to die 2 weeks after infection. Four crickets (2 male and 2

454 female) acted as sentinels. They were housed in the same room, but did not participate in

455 mating trials with infected crickets. Their fat body was collected at the same time. The

456 harvested fat body was tested for virus using PCR as described above.

457

458 Behavioural Effects

459

460 Effects of Infection on Anti-Predator Behaviour

461

462 A modified plus maze was used to assess anti-predator behaviour (see Adamo et. al.,

463 2013). Briefly, the plus maze was shaped like a plus sign and constructed of black

464 acrylic. It consisted of 4 arms (14 x 8 x 6.5 cm each) and an open central area ($8 \times 8 \times 6.5$

465   cm). During the trial, two opposing arms were covered with heavy black bristle board,

466   creating dark spaces underneath. Crickets were gently transferred from their home cage to

467   the central, uncovered area. They remained under the transfer cup for 1 min. The cup

468   was then removed and we measured the time the cricket remained motionless. We called

469   this time period 'freezing'. Freezing is a stereotypic anti-detection response associated

470   with predation avoidance across taxa (Stynoski & Noble 2012) including crickets

471   (Niemelä et al., 2012). We also measured the number of times each cricket entered an

472   arm of the plus maze as an assessment of its tendency to explore its environment. We

473   measured the amount of time each cricket spent locomoting as a proxy for behavioural

474   activity. We also measured the time it spent under covered arms to assess its tendency to

475   avoid open spaces. The apparatus was cleaned with disinfectant between trials. The

476   person scoring the trials was blind to the infection status of the cricket. There are no sex

477   differences in plus maze performance (Adamo et al., 2013); therefore data from both

478   sexes were pooled.

479

480   Effect of infection on sexual behaviour

481

482   We assessed the latency (time to singing) and duration of courtship behaviour in male

483   and female infected and uninfected crickets. Infected and uninfected crickets were

484   isolated into single containers (17cm x 15cm x 9.5cm) for at least one day prior to the

485   mating trial. Both male and female crickets were transferred to a clean, fresh container

486   (17cm x 15cm x 9.5cm) separated by a divider. After 1 min the divider was removed.

487   The time to first contact was recorded, as was the time until the male began to sing. The

488   time of each female mounting was also noted, as was the time of copulation, if any. If

489   there was no mating after 40 min, crickets were re-tested with a fresh partner for a further

490   40 min.

491

492   We also tested whether an immune challenge affected the latency of healthy male crickets

493   to produce courtship song. Male crickets were given a 2µL injection of heat-killed *S.*

494   *marcescens* (approx 1 x 10^{4}cells) in sterile water, or acted as an unhandled control.

495   Ninety minutes later, males were given mating trials with uninfected females as described

496   above. This time point was chosen because there is a clear physiological response (i.e.

497   lipid release) ninety minutes after injection of heat-killed *S. marcescens* (Adamo et al.,

498   2008).

499

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

501

502   Anecdotal observations suggested that virus-infected crickets showed normal behaviour

503   until close to death. We tested whether infected crickets could express illness-induced

504   anorexia, a robust response to immune activation in crickets (Adamo et al., 2010). The

505   experiment followed the procedure of Adamo et al., (2010). Infected crickets were

506   deprived of food, but not water, for 2 days. They were then given a 2µL injection of

507   heat-killed *S. marcescens* (approx 1 x 10^4 cells), a 2µL injection of sterile water, or acted

508   as an unhandled control. Injections of heat-killed *S. marcescens* are known to induce

509   illness-induced anorexia in this species (Adamo et al., 2010). One hour after injection,

510   crickets were placed on one side of an experimental chamber (17cm x 15cm x 9.5cm)

511   with a food pellet in the other, and the amount of time they spent feeding over the 30 min

512   trial was recorded. These times were chosen based on the earlier study (Adamo et al.,

513   2010). Adamo et al., (2010) found no effect of sex on illness-induced anorexia, so

514   information on both sexes was pooled. The results were also compared with data

515   collected on uninfected animals.

516

517   Physiological Effects – Evidence for Alteration of Immune Function.

518

519   Hemocyte Counts

520

521   Hemocyte counts were made by removing 4 µL of hemolymph from crickets using a 10

522   µL automatic pipetter and an ice-cold pipetter tip flushed with anti-coagulant (0.15 M

523   NaCl, 10 mM EDTA, 10 mM glutathione and a few crystals of phenothiocarbamide and

524   protease inhibitor cocktail (enough to form a supersaturated solution)). Hemolymph was

525   transferred to 200 µL of ice-cold anti-coagulant, gently mixed, and then a 10 µL sample

526   was placed on a Fuchs-Rosenthal counting chamber within 1 min of collection. Cells

527 were counted using phase-contrast microscopy. Hemocyte number does not differ

528 between the sexes (Adamo, 2010); therefore results from males and females were pooled.

529

530 Total Hemolymph Protein and Phenoloxidase Activity

531

532 To measure the total protein content of the hemolymph, 4µL of hemolymph was removed

533 as described previously from both infected and uninfected crickets. The hemolymph was

534 added to 60 µL of ice-cold phosphate buffered saline and immediately vortexed for 5 s.

535 10 µL of the hemolymph/saline mixture described above was added to 180 µl of Bradford

536 reagent in a 96 well plate. After a15 min incubation with the reagent, the absorbance was

537 read at 590 nm. Each assay was run with standards of bovine serum albumen.

538

539 Phenoloxidase activity was measured using a method modified from Bidochka et al.,

540 (1989). 4µL of hemolymph was removed as described previously from both infected and

541 uninfected crickets. 15 µL of the hemolymph/saline mixture (see above) was transferred

542 to a tube containing 20 µL of water. Cricket hemolymph rarely exhibits any spontaneous

543 activity (Adamo, 2004) and this tube provides a baseline measure. A further 15 µL of the

544 hemolymph/saline mixture was added to a tube of 20 µL of bovine pancreas alpha

545 chymotrypsin (1.2 mg/mL) dissolved in water. The chymotrypsin converts

546 prophenoloxidase into the active form (Adamo, 2004). This tube provides an estimate of

547 the maximal potential activity. Another 15 µL of the hemolymph/saline mixture was

548 added to 18 µL of water and 2 µL of heat-killed *S. marcescens*. This tube assesses the

549 amount of activation of prophenoloxidase during an immune challenge *in vitro*. The tubes

550 were left to incubate at room temperature for 20 min. After the incubation period, tubes

551 were centrifuged at room temperature at 10,000 for 5 min. 30 µL of supernatent was

552 added to 180 µL of 0.02 M L-Dopa in a 96 well plate. After mixing, the change in

553 absorbance at 490 nm was monitored for 20 min. Preliminary studies showed that this

554 was within the linear range of this assay. Standards using mushroom tyrosinase were run

555 as well on the same plate.

556

557 Statistics

19

558

559   Statistics were performed using IBM SPSS Statistics (ver. 19) and GraphPad

560   Prizm (ver. 5.0b, GraphPad Software, La Jolla, CA, USA). A principal components

561   analysis (PCA) was performed on the plus maze behavioural measures (locomotion, time

562   spent freezing, the number of arm entries and the time spent in the covered arms) as

563   described previously (Adamo et al., 2013). Only the first component of the PCA had an

564   eigenvalue above 1 (score=2.1) and it explained 70% of the total variance (n=23

565   crickets). The validity of the PCA was shown by the measure of sampling adequacy

566   (Kaiser-Meyer-Olkin=0.46) and a highly significant Bartletts's test of sphericity

567   (p<0.0001). The first component of the PCA was used as a summary anti-predator score.

568   Data were tested for normalacy; normally distributed data were tested with parametric

569   statistics. Non-normal data were analyzed using non-parametric statistics (Meddis,

570   1984). Non-parametric tests were also used for analyses in which at least one of the

571   treatment groups had a sample size less than 6; Meddis (1984) argue that sample sizes

572   smaller than this cannot be adequately tested for a normal distribution.

573

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575

578

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580

583

584   Author Contributions

585   S. Adamo generated the hypothesis, designed the study, analyzed the data and wrote the

586   paper. I. Kovalko helped design and perform the behavioural assays, performed the PCR

587   analysis, and critically read the paper. R. Easy designed and helped perform the PCR

588 analysis and critically read the paper. D. Stoltz performed and analyzed the EM study

589 and helped edit the paper.

590

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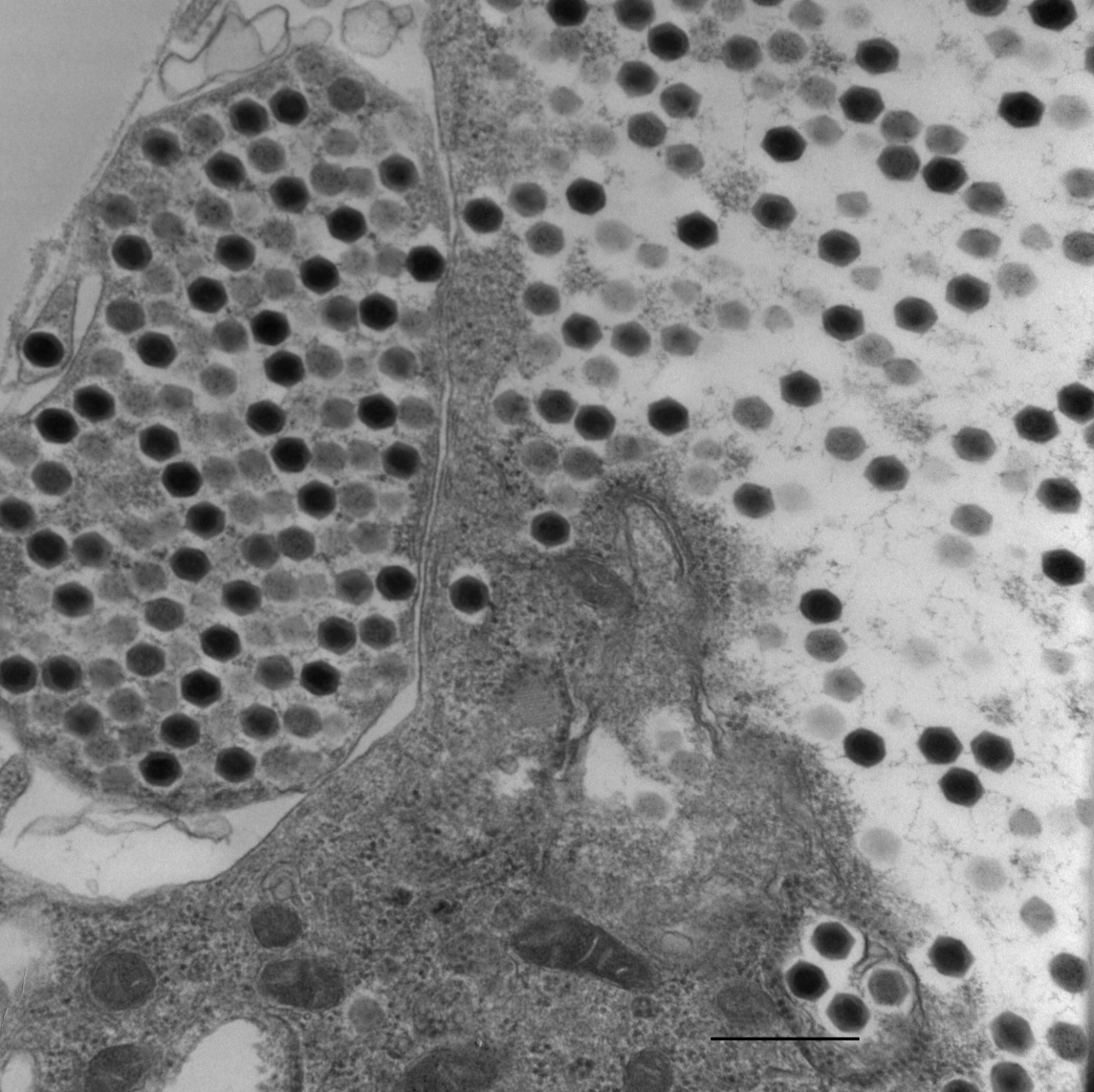
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743   Figure Legends.

744

745   Figure 1. **Cytoplasm of fat body cell infected with IIV-6/CrIV virus**. The scale bar

746   represents 500 nm.

747

748   Figure 2. **Infected males have a shorter latency to sing than controls**. The bars

749   denote means, and the error bars represent standard error of the mean. Pre-Con were

750   uninfected crickets tested prior to the infection entering the colony. Pre-Con+Sh were

751   uninfected sham challenged crickets tested prior to the infection entering the colony. Pre-

752   Con+Bac were uninfected, immune challenged crickets tested prior to the infection

753   entering the colony

754

755   Figure 3. **Infected crickets showed no evidence of illness-induced anorexia**. Bars

756   denote means and error bars denote standard error of the mean. All values were

757   normalized against the average time uninfected crickets spent feeding. Inf=infected. Pre-

758   Con were uninfected crickets tested prior to the infection entering the colony. Pre-

759   Con+Bac were uninfected, immune challenged crickets tested prior to the infection

760   entering the colony.

761

762   Figure 4. **Infected crickets had lower total phenoloxidase activity**. Bars denote means

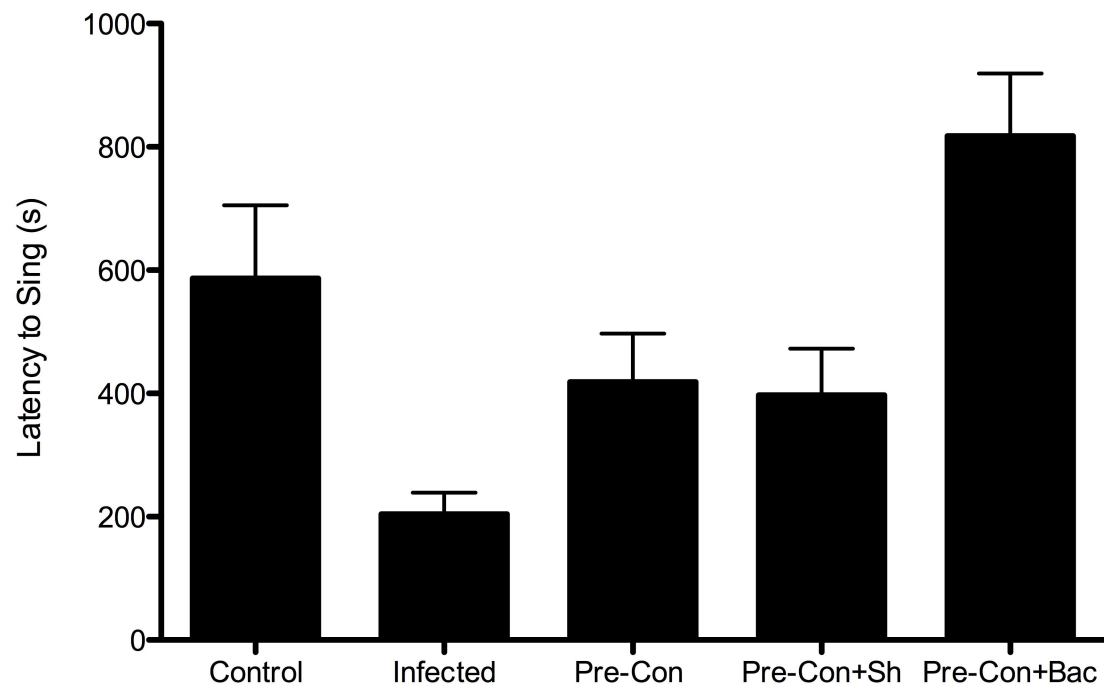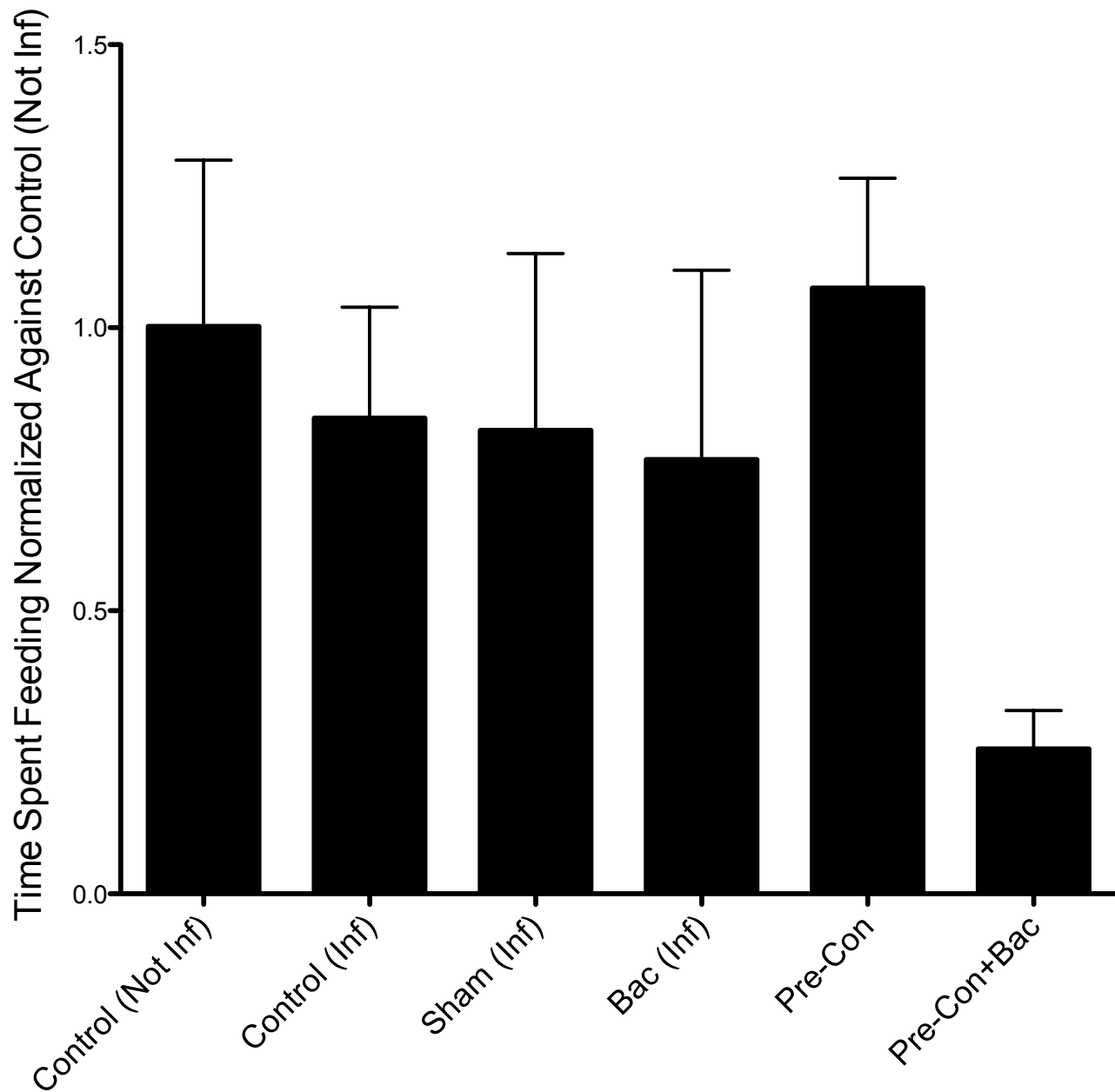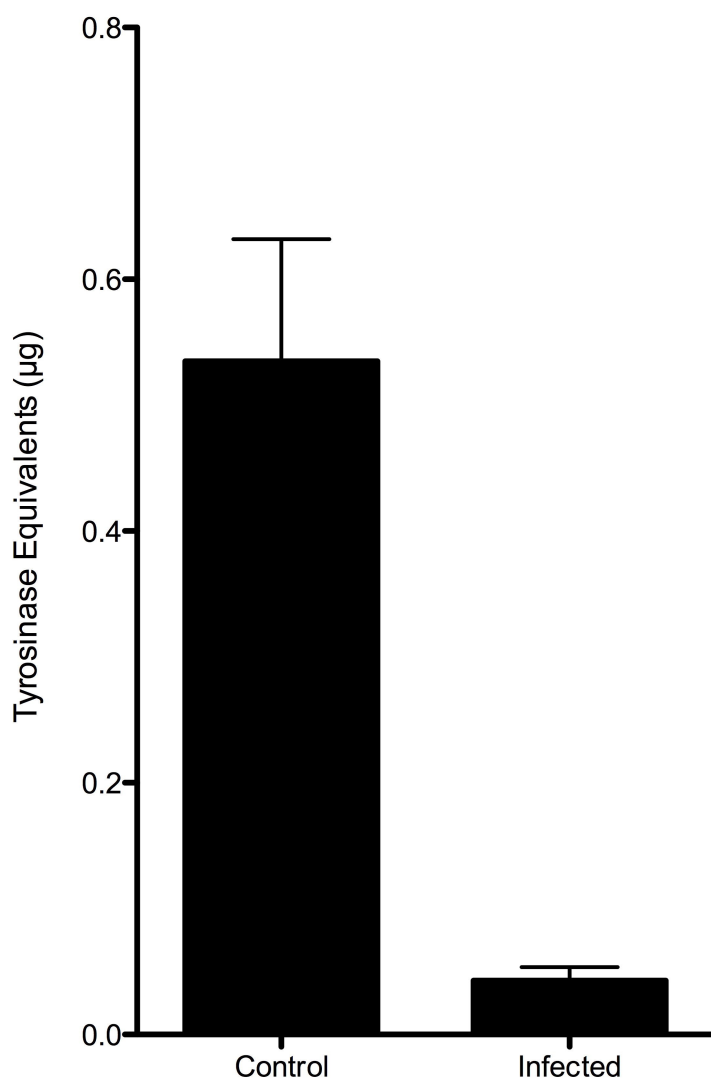763   and error bars represent standard error of the mean.

Fig. 2

Fig. 3

Fig. 4