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1 **A screen for bacterial endosymbionts in the model organisms Tribolium castaneum,**

2 **T. confusum, Callosobruchus maculatus and related species.**

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16 **Short title**: Screen for symbionts in Tenebrionidae and Bruchidae

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18

19 **Abstract**

20 Reproductive parasites such as Wolbachia are extremely widespread amongst the

21 arthropods and can have a large influence over the reproduction and fitness of their hosts.

22 Undetected infections could thus confound the results of a wide range of studies that

23 focus on aspects of host behaviour, reproduction, fitness and degrees of reproductive

24 isolation. This potential problem has already been underlined by work investigating the

25 incidence of Wolbachia infections in stocks of the model system Drosophila

26 melanogaster. Here we survey a range of lab stocks of further commonly used model

27 arthropods, focussing especially on the flour beetles Tribolium castaneum and T.

28 confusum, the cowpea weevil Callosobruchus maculatus and related species (Coleoptera:

29 Tenebrionidae and Bruchidae). These species are widespread stored product pests so

30 knowledge of infections with symbionts further has potential use in informing biocontrol

31 measures. Beetles were assessed for infection with three known microbial reproductive

32 parasites: Wolbachia, Rickettsia, Spiroplasma. Infections with some of these microbes

33 were found in some of the lab stocks studied, although overall infections were relatively

34 rare. The consequences of finding infections in these or other species and the type of

35 previous studies likely to be affected most are discussed.

36

37 **Key words:** Coleoptera, reproductive isolation, reproductive parasite, sexual conflict,

38 sexual selection, Wolbachia

39

40 **Introduction**

41 Manipulative reproductive parasites are known to be extremely common in arthropods. It

42 has recently been estimated that one of these parasites, Wolbachia, infects an astonishing

43 66% of insect species (Hilgenboecker et al., 2008). Considering that insects constitute

44 roughly 75% of global biodiversity (Grimaldi & Engel, 2005), this translates to a single

parasite infecting roughly half of the extant species worldwide, a figure that emphasises the significance of Wolbachia in terms of being an extraordinarily successful parasite (see Siozios et al., 2008). Beyond its prevalence in insects, Wolbachia is also known to infect a wide range of other animal groups, including arachnids such as mites (Breeuwer, 1997), spiders (Goodacre et al., 2006) and ticks (Noda et al., 1997). Wolbachia has also been reported to occur in crustaceans (Cordaux et al., 2001) and filarial nematodes (Taylor & Hoerauf, 1999).

Since the discovery of Wolbachia (Hertig & Wolbach, 1924) and its potential influence on host biology (Yen & Barr, 1971), other microbes have been found that have similar effects, such as Arsenophonus (Gherna et al., 1991), Rickettsia (Werren et al., 1994), Flavobacteria (Hurst et al., 1997, 1999), Cardinium (Zchori-Fein et al., 2004) and Spiroplasma (Hackett et al., 1986). So far, these bacteria have also been documented in a wide range of insects (reviewed in Hurst & Jiggins, 2000; Duron et al., 2008a), spiders (Goodacre et al., 2006, Duron et al., 2008b, Goodacre & Martin, 2013) and other arachnids (Martin & Goodacre, 2009). Together with Wolbachia, these reproductive parasites as a group potentially affect an even greater number of species overall.

Maternally inherited microbes such as Rickettsia and Wolbachia can manipulate host reproduction in various ways in order to favour their own transmission (Charlat et al., 2003; Goodacre & Martin, 2012). Horizontal transfer has been achieved through laboratory manipulations (Riegler et al., 2004) and is assumed to also occur in nature, for example via transfer between a host and a parasite (Heath et al., 1999). However, the predominant route of transmission of these bacteria is vertical, hence there can be a selective advantage to the microbe favouring a bias towards infected females in the population. Such a bias can be achieved via distorting the offspring sex ratio in favour of females via male-killing (e.g. Hackett et al., 1986), feminizing male embryos (e.g. Kageyama et al., 2002) or by inducing parthenogenesis (e.g. Arakaki et al., 2000).

71 Wolbachia has also been shown to cause cytoplasmic incompatibility (CI) altering

72 reproduction in a range of ways (Yen & Barr, 1971; Werren, 1997; Stouthamer et al.,

73 1999; Duron et al., 2008a). CI may greatly reduce fertility and even cause sterility, with

74 effects being either unidirectional (i.e. between infected and uninfected individuals), or

75 bidirectional (i.e. between individuals infected with different Wolbachia strains). Such

76 incompatibilities, especially when bidirectional, could limit gene flow amongst

77 populations of a species and can be proposed to be influential in longer term evolutionary

78 processes such as the development of reproductive isolation and, ultimately, speciation

79 (Wade & Stevens, 1985; Breeuwer & Werren, 1990; Telschow et al., 2005). Finally,

80 general effects on reproduction and fitness have also been documented (see Table 1 for

81 an overview). These effects need not be negative, indeed Wolbachia infections are shown

82 to increase resistance to particular viruses in Drosophila (Hedges et al., 2008, Osborne et

83 al. 2012) and Aedes aegypti (Bian et al., 2010).

84 It has been suggested that the finding of the intracellular bacterium Wolbachia in

85 ca. 30% of commonly used Drosophila stocks (housed at the Bloomington Drosophila

86 Stock Center) might call into question the results of many evolutionary studies (Clark et

87 al., 2005). The widespread occurrence of Wolbachia in such a ubiquitously used model

88 organism is potentially alarming because it raises the possibility that differences in

89 reproductive and/or fitness traits or compatibilities between populations might have a

90 microbial basis rather than solely be caused by other proposed mechanisms.

91 It should be noted that the situation is more complicated than merely considering

92 whether or not populations harbour Wolbachia (or any other individual reproductive

93 parasite). Seemingly 'uninfected' stocks could well harbour other bacteria that can

94 similarly affect their hosts (e.g. Cardinium, Flavobacteria, Rickettsia, Spiroplasma,

95 Arsenophonus). A survey of stocks of different Drosophila species indeed finds that

96 several species also harbour Spiroplasma (Tucson Drosophila Species Stock Center:

97   Mateos et al., 2006). Similarly extensive surveys have assessed infections with various

98   reproductive parasites in further dipteran species belonging to the superfamily Muscoidea

99   (ca. 70 species: Martin et al., 2012), and the Dolichopodidae and other Empidoidea (ca.

100   240 species: Martin et al., 2013a,b). Although Wolbachia infected flies more commonly,

101   infections with Spiroplasma, Rickettsia and Cardinium were also found (Martin et al.,

102   2012, 2013a,b). There is extensive evidence for both Wolbachia and Spiroplasma causing

103   differences in host reproduction, including in Diptera (Duron et al., 2008a). Nevertheless,

104   it is unclear how problematic the widespread infections in Drosophila stocks (Clark et al.,

105   2005) actually are to the evolutionary studies carried out on them. How robust are

106   conclusions drawn from previous experiments where the bacterium might – or might not -

107   have been present? Here we review the type of experiments or traits under study that are

108   likely to be most susceptible.

109   Considering how widespread such bacterial endosymbionts are among arthropods

110   (Goodacre et al., 2006; Duron et al., 2008a; Hilgenboecker et al., 2008), and that

111   Wolbachia is not the only microbe known to have such effects, we include in our study

112   data from a range of model systems where we establish the presence of Wolbachia and of

113   other microbes that are similarly implicated in altering the biology of their hosts.

114   Conceivably, the presence of such parasites will be most relevant in model systems used

115   extensively for studies on reproduction. Beyond Drosophila melanogaster, other lab

116   organisms, which have (among other things) been used frequently for investigating

117   reproductive biology, are beetles belonging to the genus Tribolium (Tenebrionidae). The

118   red flour beetle T. castaneum is a widespread pest and has become a major model system

119   for the study of pre- and postcopulatory sexual selection (Fedina & Lewis, 2008; Pai &

120   Bernasconi, 2008; Michalczyk et al., 2010; Sbilordo et al., 2011; Grazer & Martin, 2012),

121   and sexual conflict (Michalczyk et al., 2011a). This promiscuous species has also been

122   used to assess the causes and consequences of polyandry, with recent examples focussing

123   on the roles played by inbreeding (Michalczyk et al., 2011b) and environmental change

124   (Grazer & Martin, 2012). T. castaneum is also an important model in the study of host-

125   parasite conflicts and immunity (e.g. Blaser & Schmid-Hempel, 2005; Zou et al., 2007;

126   Wegner et al., 2008, 2009; Bérénos et al., 2009; Kerstes et al., 2013; Hangartner et al.,

127   2013). Similar to D. melanogaster, one of the attractions of this system is the access to

128   molecular tools such as the sequence of the entire genome of T. castaneum (Richards et

129   al., 2008). Stocks of the related confused flour beetle T. confusum are already known to

130   commonly harbour Wolbachia, with the microbe causing CI in this species (Fialho &

131   Stevens, 1996). Interestingly, this CI-inducing Wolbachia strain is genetically

132   indistinguishable (based upon sequences from four gene regions) from that infecting the

133   congeneric species T. madens where it causes male killing (Fialho & Stevens, 2000).

134   Callosobruchus (Bruchidae) seed beetles are pests of stored legumes and can be

135   easily reared in the lab. These species have also been the subject of intense study in the

136   context of postcopulatory sexual selection (e.g. Wilson et al., 1997; Eady et al., 2004;

137   Maklakov & Fricke, 2009), sexual conflict (Crudgington & Siva Jothy, 2000; Arnqvist et

138   al., 2005; Rönn et al., 2007, 2011), and reproductive isolation (Fricke & Arnqvist, 2004),

139   including targeted experimental evolution studies (e.g. Fricke & Arnqvist, 2007; Gay et

140   al., 2009; Maklakov et al., 2009, 2010). Callosobruchus has also been the focus of

141   detailed studies of the fitness consequences of ageing and inbreeding (Fox et al., 2004,

142   2006, 2011a,b; Bilde et al., 2009) including relationships with the environmental context

143   (Messina & Fry, 2003; Fox et al., 2011b). Finally, many of the above representatives of

144   the bruchid and tenebrionid beetles and closely related species are widespread pests of

145   stored products. This adds an applied perspective, as Wolbachia has been discussed

146   intensively as a potentially useful ally in the fight against pests and vectors of disease, for

147   instance of mosquitos (see Laven, 1967; reviewed in Iturbe-Ormaetxe et al., 2011) or

148   medflies (Zabalou et al., 2009). More generally, greater consideration of impacts of

149 symbionts on insect pests and vectors has been argued to be critical in assessing risks and

150 effectiveness of biocontrol measures (Zindel et al., 2011).

151   In summary, the aims of the present study were two-fold: a) to survey a broad

152 selection of commonly used laboratory strains of Tribolium and Callosobruchus beetles

153 and related species for infection with three microbial reproductive parasites (Rickettsia,

154 Spiroplasma and Wolbachia), and b) to assess consequences of finding such infections in

155 these or other species and discuss the type of previous evolutionary study and data most

156 likely to be at risk from the confounding effects of endosymbiont infections.

157

158 **Materials and methods**

159 Prior to testing, all the tenebrionid beetle stocks assayed in the present study had been

160 maintained at large population sizes and housed on organic flour (with 10% brewer's

161 yeast) in dark climate chambers at a constant 30 ° C (as standard for the stocks used, see

162 Grazer & Martin, 2012). Although higher rearing temperatures are frequently used for

163 tenebrionids, temperatures of above ca. 36 degrees are known to impact on endosymbiont

164 infections, thus stocks that had been kept under these conditions in the past were avoided

165 in our study (see e.g. Sakamoto et al., 2008). Bruchid beetle stocks were held in climate

166 chambers at constant conditions of 27°C and 45% (± 10%) relative humidity under a

167 12:12 h light-dark cycle. Beetles were held in 1L glass jars and maintained at large

168 population sizes of 250-300 beetles per generation and provided with excess amounts of

169 black-eyed beans (Vigna unguiculata).

170

171 Sample beetles for PCR testing were removed from their stock containers and

172 subsequently kept in 70% ethanol until DNA extractions. DNA was extracted from

173 abdominal tissue using QIAGEN DNEasy kits and eluted in 100 µl distilled water. The

174 success of DNA extraction was established by polymerase chain reaction (PCR) using

175 host-specific primers designed to amplify a fragment of the mitochondrial cytochrome

176 oxidase I (COI) gene (tenebrionid beetles) and a nuclear microsatellite dimer repeat

177 (Callosobruchus beetles) respectively. (COI primers: Co12309 5' TTT ATG CTA TAG

178 TTG GAA TTG G 3' and Co12776 5'GGA TAA TCA GAA TAT CGT CGA GG, as

179 described in Hedin & Maddison 2001; Callosobruchus microsatellite primers: 5'ATG

180 GCG ATT GCT ATT CTG TTG -3 and 5' AAA TAA CAG GCA TCA AAA CAA CAT

181 3' (Fricke et al. unpublished). Amplification of host DNA was obtained from all our

182 samples indicating that DNA extraction had been successful. Samples were subsequently

183 tested by PCR for Wolbachia , Rickettsia spp. and Spiroplasma spp. using the methods

184 previously described by Majerus et al. (2000) which were as follows: i) A section of the

185 Wolbachia cell surface protein gene wsp was amplified using WSP-F (5′ -

186 TGGTCCAATAAGTGATGAAGAAACTAGCTA-   3′)   and   WSP-R   (5′   -

187 AAAAATTAAACGCTACTCCAGCTTCTGCAC- 3′ (Jeyaprakash & Hoy, 2000). ii) A

188 section of the citrate gene in Rickettsia spp was amplified using RICS741F (5 ′ -

189 CATCCGGAGCTAATGGTTTTGC-   3′)   and   RCIT1197R   (5′   -

190 CATTTCTTTCCATTGTGCCATC- 3′ (Davis et al., 1998). (iii) A section of the

191 intergenic ribosomal spacer of the Spiroplasma ixodetis group was amplified using Spits-

192 J04 (5 ′ -GCCAGAAGTCAGTGTCCTAACCG-3 ′) and Spits-N55 (5 ′ -

193 ATTCCAAGGCATCCACCATACG-3 ′ (Majerus et al. 1999). All PCRs were carried

194 out in an MJ cycler in a total volume of 25 µL containing 1 unit of Taq, 2.5 mM MgCl 2 ,

195 0.5 mM of each dNTP, 400 nM of each primer and 1 µL of DNA solution, in a buffer of

196 10 mM Tris-HCl, 50 mM KCl pH 8.3 (20 ° C). An initial denaturation at 94 ° C for 1 min

197 was followed by 35 cycles of 94 ° C for 30 s, 55 ° C (endosymbiont genes) or 50 ° C

198 (COI gene) or 53 ° C (Callosobruchus microsatellite) for 20 s and 72 ° C for 30 s. Bands

199 were visualized by gel electrophoresis on a 1.5% agarose gel stained with ethidium

200 bromide All PCRs were run in the presence of both positive and negative controls. The

201 list of stocks tested for presence of Rickettsia, Spiroplasma & Wolbachia can be found in

202 Table 2 and associated footnotes.

203

204 **Results**

205

206 **Tenebrionidae**

207 The results of our PCR survey for infections with the three endosymbionts are displayed

208 in Table 2. Results confirm the presence, as expected, of Wolbachia in T. confusum,

209 where it has previously been shown to cause CI (Wade & Stevens, 1985). In all T.

210 confusum strains except HP70 both males and females were positive for Wolbachia.

211 Previous studies have indicated that separate stocks may harbour identical (or at least

212 compatible) Wolbachia strains (Fialho & Stevens, 1996). Preliminary crosses between

213 infected and uninfected stocks appear to confirm this result (Martin, unpublished data). In

214 contrast with T. confusum, individuals from the large number of T. castaneum strains

215 tested were all apparently devoid of Wolbachia infections. Whereas in the former six out

216 of eight stocks tested positive for Wolbachia, in contrast none of the ca. 40 T. castaneum

217 strains tested appeared to be infected, although three of these were found to carry

218 Spiroplasma, and one harboured Rickettsia (for details see Table 2). The closely related

219 species T. freemani also appeared to be free of Wolbachia infection as was the single

220 strain of T. madens tested in this survey. Others have shown that T. madens can be

221 infected with Wolbachia strains genetically indistinguishable from that infecting T.

222 confusum and that the bacterium distorts sex ratio by causing male-killing (Fialho and

223 Stevens 2000). In accordance with the lack of infection in this study, no bias in sex ratio

224 was apparent in the stock tested here (Martin, personal observation). Similarly, no

225 Wolbachia infections were found in the remaining congeneric species (T. anaphe, T.

226 audax, T. brevicornis or T. destructor) or any of the other tenebrionid species tested

227 (Gnatocerus cornutus, Latheticus oryzae or Palorus ratzeburgii) although we note that

228 the number of samples tested for these species was very small (only a single individual in

229 some cases) and thus our power to detect endosymbionts that are at anything less than

230 100% prevalence was low. Tests for Rickettsia and Spiroplasma detected neither of these

231 types of bacteria in any of the individuals tested.

232

233 **Bruchidae**

234 Results are presented in Table 3. Testing 16 different populations of Callosobruchus

235 maculatus and two related species C. rhodesianus and C. analis shows generally very low

236 infection rates. Spiroplasma could not be detected from any of the samples while

237 Wolbachia was only found in one individual out of four tested in C. rhodesianus. All

238 three species C. maculatus, C. rhodesianus and C. analis show single infections with

239 Rickettsia.

240

241 **Discussion**

242 Tests for endosymbiotic bacteria in the tenebrionid and bruchid beetles in this study

243 appear to indicate that symbionts may be less common in these groups than in the insects

244 assessed previously (Hilgenboeker et al., 2008). The overwhelming majority of currently

245 available data are from studies on the interaction of insect hosts with Wolbachia, with far

246 less being known about effects of other endosymbionts (examples in Table 1). Of the four

247 classic phenotypes (CI, male-killing, feminization and parthenogenesis), all have been

248 documented in a range of host species for Wolbachia and a few of these have also been

249 shown to be caused by infections with other known endosymbionts. In Tribolium spp.

250 specifically, research has focussed solely on Wolbachia, with evidence to indicate that

251 this symbiont causes CI in T. confusum and male-killing in T. madens (Fialho & Stevens,

252 2000). Further impacts on non-reproductive traits are also possible as evidenced by recent

253 work suggesting a negative effect of Rickettsia infection on long-distance dispersal

254 behaviour in a spider (Goodacre et al., 2009).

255   Precisely to what degree endosymbiont infections could confound results obtained

256 from lab populations will depend on how the microbe affects the host. For example, if CI-

257 causing bacteria remain undetected in particular insect stock populations, this could

258 compromise studies involving inter-population crosses. Furthermore, if the stock

259 populations in question are not uniformly infected, it could also explain differential

260 reproductive successes across studies of single populations. Temporal changes in

261 reproductive success of single populations might also occur if the natural rate of bacterial

262 transmission from mother to offspring is altered under laboratory conditions, such that

263 populations experience rapid changes in the frequency of endosymbiont infections after

264 only a few generations in the lab. Such issues could be especially problematic when

265 assessing reproductive isolation using postzygotic measures, as is often the case in studies

266 directed towards understanding processes such as genetic isolation and speciation.

267 Prezygotic measures could also be confounded if infection status affects mate preferences

268 (see e.g. Markov et al., 2009) or the frequency of mating (Champion de Crespigny et al.,

269 2006) (see also Table 1). It seems perhaps less likely that phenotypes involving sex ratio

270 skew, such as parthenogenesis, feminization or male killing could 'silently' affect

271 experimental populations. A strong bias towards females might appear likely to be picked

272 up during routine work, although actual protocols used would need to be evaluated to

273 assess possible risks of missing skewed sex ratios.

274   More general and less drastic negative (or positive) effects, for example on fitness

275 are perhaps less likely to be an issue. Here it is unclear whether one could argue that

276 patterns would be majorly influenced by undetected endosymbionts, unless populations

277 used are not uniformly infected. Laboratory populations will also be, or have been,

278 affected by a large range of other intrinsic and extrinsic factors. These remain for the

279 most part equally silent, and may for example include nematodes, mites, other pathogens

280 or parasites, or selfish genetic elements such as Medea in T. castaneum (Lorenzen et al.,

281 2008). In this respect, reproductive parasites are probably not truly a greater challenge

282 than any other of these unknowns, which already have to be taken into account.

283   Artificial transfer experiment protocols exist for Tribolium beetles (Chang &

284 Wade, 1996), potentially offering a controlled way to assess effects on existing (or novel)

285 hosts experimentally. Indeed, the effects on reproduction of the various symbionts remain

286 largely unresolved for many populations (or species). Reproductive parasites can

287 specifically impact on reproductive traits (see Table 1), so beyond obvious involvement

288 in conflict between host and symbiont, they can impinge on sexual conflict between

289 males and females (see Martin & Gage, 2007). A promising and targeted means of

290 illuminating the separate and combined action of these (interspecific and intraspecific)

291 evolutionary conflicts would be to use a combined experimental evolution approach akin

292 to previous experiments focusing on either sexual conflict (e.g. Martin & Hosken, 2003,

293 2004; Fricke & Arnqvist, 2007; Gay et al., 2009; Hosken et al., 2009; Maklakov et al.,

294 2010; Michalczyk et al., 2011a) or host-parasite conflict (e.g. Bérénos et al., 2009).

295 Findings of experimental evolution studies in Tribolium and Callosobruchus (e.g. Gay et

296 al., 2009; Maklakov et al., 2009, 2010; Michalczyk et al., 2011a) coupled with detailed

297 knowledge of reproduction in these study systems could provide a solid base for

298 understanding interactions between hosts and their reproductive parasites.

299   One means of assessing symbiont effects has been to treat animals with antibiotics

300 to cure them of their infections. However, treatment with this antibiotic also has the

301 potential to influence other fitness traits and likely eliminates other known or unknown

302 bacteria with unpredictable consequences. Furthermore, there are potentially other

303 confounding effects, such as persistent effects on metabolism, after curing with

304 Tetracycline (see e.g. Ballard & Melvin, 2007).

305   Infections with Rickettsia and Spiroplasma are found across a wide range of

306   arthropods so were hence also tested for in this study in addition to Wolbachia. In fact,

307   multiple infections within species or groups of species are not uncommon (e.g. Weeks et

308   al., 2003; Goodacre et al., 2006). In this study we only found very few infected

309   individuals and only one multiply infected female (C. rhodesianus infected with both

310   Wolbachia and Rickettsia, in contrast with Kondo et al., 1999; see Table 3). More

311   generally, though, further complications could arise if different infections interact with

312   one another. Such inter-microbial interactions may be a promising area of future research

313   (see e.g. Engelstädter et al., 2008).

314   Clearly evolutionary biologists need to be aware of the complex relationship

315   between a study organism and its associated symbionts or parasites. Studies such as this

316   or the large-scale work already undertaken on Drosophila (Clark et al., 2005, Mateos et

317   al., 2006) and other Diptera (Martin et al., 2012) can only be informative. Researchers

318   should be grateful rather than alarmed that leading lab 'work-horses' such as Drosophila,

319   Tribolium or Callosobruchus are not impervious to the range of microbial diversity

320   commonly found in the wild. For a start, the majority of arthropod species are likely to

321   have evolved in contact with Wolbachia, so study organisms infected with this parasite

322   are probably more representative of the situation in the wild. Moreover, this should really

323   be seen as a valuable opportunity to address pressing questions in a burgeoning area of

324   research, using the well-understood systems that model lab organisms such as Tribolium

325   provide. Here one can draw not only upon a wealth of extensive and highly relevant

326   information on host reproduction but also access the full array of genetic tools available

327   for these species.

328   To conclude, we provide data on infections with three common reproductive

329   parasites in stock populations of the popular model systems T. castaneum and C.

330   maculatus and a range of related species. We confirm an emerging pattern where

331   Wolbachia infections are widespread in T. confusum stocks, yet the same types of

332   bacteria (i.e. those that are sensitive to our detected methods) appear to be conspicuously

333   absent in other Tenebrionidae assessed (see also Chang & Wade, 1996; Kageyama et al.,

334   2010). Additionally, our results confirm a lack of Wolbachia infections in C. maculatus

335   matching previous surveys (Kondo et al., 1999; Kageyama et al., 2010). In contrast,

336   Wolbachia has previously been documented in C. analis and C. chinensis (Kageyama et

337   al., 2010). However, symbionts other than Wolbachia were not assessed in previous

338   surveys where tenebrionid or bruchid host species were included (e.g. Kageyama et al.,

339   2010). Here, C. maculatus is found to harbour infections with Rickettsia, illustrating the

340   point that assessing several symbionts is worthwhile (this also holds for T. castaneum, see

341   Table 2).

342   It is important to emphasise that our failure to detect bacterial DNA in

343   particular species/stocks included in this study does not imply that these are necessarily

344   endosymbiont free. It only implies that the individuals that we tested do not carry

345   bacterial strains that we can detect and we note the number of individuals that we have

346   tested in our study is small. Low prevalence of endosymbionts, such as male killers,

347   (which may have a lower prevalence within a population than their CI-inducing

348   counterparts) within a population or very low bacterial titres would make it less likely

349   that they would be detected in our study. Furthermore, divergent bacterial strains can

350   remain undiagnosed even if at high prevalence and/or high titre if they are not detected by

351   our PCR methods (e.g. as demonstrated by Simões et al. 2011). The use of next

352   generation sequencing technology to sequence all those bacteria found within in

353   combination with more comprehensive sampling may be a useful step forward in the

354   study of endosymbionts in model lab organisms, as has been applied for other

355   invertebrate groups (e.g. Kautz et al. 2013.)

356

357   Overall, we suggest that the widespread distribution of reproductive parasites in

358 lab stocks is not by itself a basis for universal concern. Clearly, however, earlier

359 interpretations should always be open to additional scrutiny or re-evaluation if necessary,

360 i.e. if stocks are infected. As a case in point, we find that the T. castaneum source

361 population used in several recent studies (Morrow et al., 2003; Michalczyk et al., 2010,

362 2011a,b; Sbilordo et al., 2011; Grazer & Martin, 2012; Hangartner et al., 2013) is free of

363 infection with the symbionts assessed. We further propose that valuable new insights

364 could be gained by considering new data on bacterial infections including all known

365 reproductive parasites in further hosts. This may be particularly useful in model systems

366 for sexual selection and related themes such as the genera Tribolium and Callosobruchus

367 where extensive knowledge of reproduction is already available. Finally, more detailed

368 knowledge accrued concerning infections can help build strong foundations for mounting

369 biocontrol measures against target taxa (see e.g. Xi et al., 2005).

370

378

379 **Disclosure**

380 The authors declare that they have no conflict of interests.

381

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Table 1. Potentially confounded reproductive traits and other measures affected by

infection with microbial reproductive parasites. Examples of symbionts causing

effects on reproduction in a range of arthropods are included with references for each trait

in the list. Wolbachia dominates in these examples as the majority of research to date has

focussed on effects of this microbe on hosts. The examples cited by no means represent

an exhaustive list.

Measures of interest	Effects	Example - symbiont: species (reference)
Reproductive isolation		
Prezygotic (mate choice)	Infection status affects assortative mating	Wolbachia: Drosophila melanogaster (Markov et al. 2009)
Postzygotic (inter-population compatibility)	Pattern indicative of postzygotic reproductive isolation between populations could be due to **cytoplasmic compatibility**	Wolbachia: Tribolium confusum (Wade and Stevens 1985)
Reproductive traits		
FEMALES		
Female fecundity	Infection associated with decrease	Wolbachia: Tribolium confusum (Wade and Chang 1995)
Oviposition behaviour	Infected females aggregate offspring (to promote matings between siblings?)	Wolbachia: Tetranychus urticae (Vala et al. 2004)
MALES		
Male fertility	Infection associated with increase	Wolbachia: Tribolium confusum (Wade and Chang 1995)
Sperm competitive ability	Infection associated with reduced sperm competition success	Wolbachia: Drosophila simulans (Champion de Crespigny and Wedell, 2006)
Male mating rate	Infected males mate at higher rates than uninfected counterparts	Wolbachia: Drosophila melanogaster & D. simulans (Champion de Crespigny et al. 2006)
Sex ratio distortion		
Apparent parthenogenesis	Could have microbial cause instead of other interpretations	Wolbachia: Encarsia formosa (Zchori-Fein et al. 1992)

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Skewed sex ratios	Decreased numbers of males in a population could be caused by **feminization**…	Wolbachia: Ostrinia furnacalis (Kageyama et al. 2002)
	… or could be due to **male-killing**	Flavobacteria: Adonia variegata (Hurst et al. 1999)
Non-reproductive traits		
Dispersal behaviour	Infected females are less likely to disperse	Rickettsia: Erigone atra (Goodacre et al. 2009)
Survival	Infection increases longevity	Wolbachia: Drosophila melanogaster (Fry and Rand 2002)
Thermotolerance	Infection increases tolerance to heat shock	Rickettsia: Bemisia tabaci (Brumin et al. 2011)

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Table 2. Overview of results from PCR screens for microbial reproductive parasites in 11 tenebrionid species. Beetle stocks were screened for infection with the 3 endosymbionts Wolbachia, Rickettsia and Spiroplasma using PCR: '+' indicates positive infection status. Samples include individuals from numerous strains of the extensively used sexual selection model system Tribolium castaneum, T. confusum and related species. F = female, M = male. Stock names and locations are provided where known.

Notes: **1)** Identity of T. castaneum stocks tested with geographical origin and year of collection if known. Strains tested were generally negative for all microbes unless otherwise mentioned in the Table: **Australia**: CTC-485 (Brisbane, 1965), GW-3 (Turner, 1988), Oz (collected in 2000) **/ Bangladesh**: Bang-2 (Dhaka, 1979), BT-15 (Dakar, 1981) **/ Brazil**: BRZ-4 (Aracatuba, 1987) **/ Canada:** Montreal (1973), NDJ-13 (Vancouver, 1976) **/ China:** Mek-1 (1987), PRC-Nan (Nanjing, 1989), PRC-Ning (Ningbuo, 1989) **/ Colombia:** COL-1 (Palmira, 1987) **/ India;** Dwi-1 (1989), PS-129 (1984), RINI-3 (Kanpur, 1989) **/ Israel:** ISR-1 (Tel Aviv, 1988), Solet (1979) **/ Japan:** Japan 2 (Kyushu Island, 1988) **/ Pakistan:** PAK-1 (Peshawar, 1979), PAK-3 (Peshawar, 1988) **/ Poland:** Pruz + (1988), Pruz 1 (1963) **/ Singapore:** HO-TCS (Senoko, 1989) **/ Thailand:** Heng-5 (Chiang Mai province, 1989) **/ Uganda:** Ug-1 (Kampala, 1989) **/ UK:** BRZ-6 (London, 1943), FSS2 (London, 1943) **/ USA:** BRM (Texas, 1988), Causey-S (South Carolina, 1991), Ga1 (Georgia, 1980), Lab S (Kansas, 1976), Little Rock (Arkansas, 1988), NDJ-11 (Hawaii, 1976), Ramsey (Minnesota, 1988), Waunakee (Wisconsin, 1992), Z-1 (Alabama, 1988) / **Mutant stocks:** fta, pygmy, Goliath, Reindeer. **2)** All beetles were kindly provided by Richard Beeman (USDA), apart from those marked with '*' provided by the Central Science Laboratory (CSL), Slough, United Kingdom and Oz provided by Tom Tregenza. For each stock extracted DNA from between 2 and10 beetles of the same sex was combined and the combined DNA tested for the presence of bacterial DNA (i.e. males and females combined separately). The exceptions to this were T. brevicornis, T. freemani and L. oryzae where only a single individual (a female) was used.

Species	Stock	Location	Wolbachia	Rickettsia	Spiroplasma
Gnatocerus cornutus	(USDA)		-	-	-
Latheticus oryzae	(USDA)		-	-	-
Palorus ratzeburgii	(USDA)		-	-	-
Tribolium anaphe *	(CSL)		-	-	-
Tribolium audax *	(CSL)		-	-	-
Tribolium brevicornis	(USDA)		-	-	-
Tribolium destructor *	(CSL)		-	-	-
Tribolium castaneum	BRZ-6	London, 1943	-	**+in (F)-**	-
	Dachshund	Mutant	-	-	**+ (M, F)**
	LabS	Kansas, 1976	-	-	**+ (F only, not detected in M)**
	Reindeer	Mutant	-	-	**+(M, F)**
	remaining stocks [1]	-	-	-	-
Tribolium confusum	HP70	Kansas, ca. 1986	-	-	-
	b,au, lod, p	Mutant	+	-	-
	Cx(apt),A(mas),mxp(Stb)	Mutant	+	-	-
	Ibad 2	Pakistan, 1988	+	-	-
	MN61	Kansas, ca. 1986	+	-	-
	PRC	China	-	-	-
	PRC-Ning	China, 1989	+	-	-
	Thai B	Thailand	-	-	-
	Ug-2	Uganda, 1989	+	-	-
Tribolium freemani	(USDA)	Japan	-	-	-
Tribolium madens	(USDA)	Kansas	-	-	-

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727 **Table 3. Overview of results from PCR screens for microbial reproductive parasites**

728 **in 3 bruchid beetle species.** Callosbruchus beetle stocks were screened for infection

729 with the 3 endosymbionts Wolbachia, Rickettsia and Spiroplasma using PCR: '+'

730 indicates positive infection status and '-' no infection. N=1 individual per sample.

731 Location names (where known) indicate where beetles were sampled.

	Location	Sex	Wolbachia	Rickettsia	Spiroplasma
C. maculatus	Brazil (USA)	F	-	-	-
	Mali	F	-	-	-
	Yemen	M	-	-	-
	IITA	M	-	-	-
	Oman	F	-	-	-
	California	F	-	-	-
	Upper Volta	Unknown	-	+	-
	South India (USA)	M	-	-	-
	Benin	F	-	-	-
	Nigeria/Lossa	M	-	-	-
	Brazil (Leicester)	F	-	-	-
	Nigeria/Zaira	M	-	-	-
	Nigeria mix	F	-	-	-
	Poly B 4	F	-	+	-
	Nigeria/Oyo	M	-	-	-
	Black	F	-	-	-
C. rhodesianus	Swaziland	M	-	-	-
	Swaziland	F	-	-	-
	Swaziland	F	+	+	-
	Swaziland	M	-	-	-
C. analis		F	-	+	-
		M	-	-	-
		F	-	-	-
		F	-	-	-

732

733 Notes: The Brazil and South India strains were split and held in different laboratories (by G. Keeney (USA)

734 and R. Smith (Leicester)) - details of their history can be found in Fricke and Arnqvist 2004. The origins of

735 the Nigeria mix and Poly B 4 strains can be found in Fricke and Arnqvist (2007) (for other stocks and

736 further details see: Giga & Smith, 1991, Rönn et al., 2007, 2011, Rankin & Arnqvist, 2008).