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1 **A screen for bacterial endosymbionts in the model organisms *Tribolium castaneum*,**
2 ***T. confusum*, *Callosobruchus maculatus* and related species.**

3

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15

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

17

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

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

52 Since the discovery of Wolbachia (Hertig & Wolbach, 1924) and its potential
53 influence on host biology (Yen & Barr, 1971), other microbes have been found that have
54 similar effects, such as *Arsenophonus* (Gherna et al., 1991), *Rickettsia* (Werren et al.,
55 1994), *Flavobacteria* (Hurst et al., 1997, 1999), *Cardinium* (Zchori-Fein et al., 2004) and
56 *Spiroplasma* (Hackett et al., 1986). So far, these bacteria have also been documented in a
57 wide range of insects (reviewed in Hurst & Jiggins, 2000; Duron et al., 2008a), spiders
58 (Goodacre et al., 2006, Duron et al., 2008b, Goodacre & Martin, 2013) and other
59 arachnids (Martin & Goodacre, 2009). Together with Wolbachia, these reproductive
60 parasites as a group potentially affect an even greater number of species overall.

61 Maternally inherited microbes such as *Rickettsia* and Wolbachia can manipulate
62 host reproduction in various ways in order to favour their own transmission (Charlat et
63 al., 2003; Goodacre & Martin, 2012). Horizontal transfer has been achieved through
64 laboratory manipulations (Riegler et al., 2004) and is assumed to also occur in nature, for
65 example via transfer between a host and a parasite (Heath et al., 1999). However, the
66 predominant route of transmission of these bacteria is vertical, hence there can be a
67 selective advantage to the microbe favouring a bias towards infected females in the
68 population. Such a bias can be achieved via distorting the offspring sex ratio in favour of
69 females via male-killing (e.g. Hackett et al., 1986), feminizing male embryos (e.g.
70 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; Béréños 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 (Crudginton & 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% (\pm 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 μ l 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 TGGTCCAATAAGTGATGAAGAACTAGCTA- 3') and WSP-R (5' -
187 AAAAATTAACGCTACTCCAGCTTCTGCAC- 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 CATTCTTTCCATTGTGCCATC- 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' -GCCAGAAGTCAGTGTCTAACCG-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 μ L containing 1 unit of Taq, 2.5 mM MgCl₂ ,
195 0.5 mM of each dNTP, 400 nM of each primer and 1 μ L 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. Béréños 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

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378

379 **Disclosure**

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

381

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692

693 **Table 1. Potentially confounded reproductive traits and other measures affected by**
 694 **infection with microbial reproductive parasites.** Examples of symbionts causing
 695 effects on reproduction in a range of arthropods are included with references for each trait
 696 in the list. *Wolbachia* dominates in these examples as the majority of research to date has
 697 focussed on effects of this microbe on hosts. The examples cited by no means represent
 698 an exhaustive list.

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

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

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700

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

707 Notes: **1)** Identity of *T. castaneum* stocks tested with geographical origin and year of collection if known.
708 Strains tested were generally negative for all microbes unless otherwise mentioned in the Table: **Australia:**
709 CTC-485 (Brisbane, 1965), GW-3 (Turner, 1988), Oz (collected in 2000) / **Bangladesh:** Bang-2 (Dhaka,
710 1979), BT-15 (Dakar, 1981) / **Brazil:** BRZ-4 (Aracatuba, 1987) / **Canada:** Montreal (1973), NDJ-13
711 (Vancouver, 1976) / **China:** Mek-1 (1987), PRC-Nan (Nanjing, 1989), PRC-Ning (Ningbo, 1989) /
712 **Colombia:** COL-1 (Palmira, 1987) / **India:** Dwi-1 (1989), PS-129 (1984), RINI-3 (Kanpur, 1989) / **Israel:**
713 ISR-1 (Tel Aviv, 1988), Solet (1979) / **Japan:** Japan 2 (Kyushu Island, 1988) / **Pakistan:** PAK-1
714 (Peshawar, 1979), PAK-3 (Peshawar, 1988) / **Poland:** Pruz + (1988), Pruz 1 (1963) / **Singapore:** HO-TCS
715 (Senoko, 1989) / **Thailand:** Heng-5 (Chiang Mai province, 1989) / **Uganda:** Ug-1 (Kampala, 1989) / **UK:**
716 BRZ-6 (London, 1943), FSS2 (London, 1943) / **USA:** BRM (Texas, 1988), Causey-S (South Carolina,
717 1991), Ga1 (Georgia, 1980), Lab S (Kansas, 1976), Little Rock (Arkansas, 1988), NDJ-11 (Hawaii, 1976),
718 Ramsey (Minnesota, 1988), Waunakee (Wisconsin, 1992), Z-1 (Alabama, 1988) / **Mutant stocks:** fta,
719 pygmy, Goliath, Reindeer. **2)** All beetles were kindly provided by Richard Beeman (USDA), apart from
720 those marked with ‘*’ provided by the Central Science Laboratory (CSL), Slough, United Kingdom and Oz
721 provided by Tom Tregenza. For each stock extracted DNA from between 2 and 10 beetles of the same sex
722 was combined and the combined DNA tested for the presence of bacterial DNA (i.e. males and females
723 combined separately). The exceptions to this were *T. brevicornis*, *T. freemani* and *L. oryzae* where only a
724 single individual (a female) was used.

725

Species	Stock	Location	Wolbachia	Rickettsia	Spiroplasma
Gnatoceus 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	-	-	-

726

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).