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

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4 **Sara L. Goodacre¹, Claudia Fricke² and Oliver Y. Martin³ ***

5 *¹School of Biology, University of Nottingham, Queen's Medical Centre, Nottingham NG7*

6 *2UH, United Kingdom;*

7 *²Institute for Evolution and Biodiversity, University of Muenster, Hüfferstr. 1, D-48 149*

8 *Muenster, Germany;*

9 *³ETH Zürich, Experimental Ecology, Institute for Integrative Biology, D-USYS, CHN E*

10 *19.2, Universitätsstrasse 16, CH-8092 Zürich, Switzerland*

11

12 *Correspondence: Oliver Y. Martin

13 email: oliver.martin@env.ethz.ch

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

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Abstract

Reproductive parasites such as *Wolbachia* are extremely widespread amongst the arthropods and can have a large influence over the reproduction and fitness of their hosts. Undetected infections could thus confound the results of a wide range of studies that focus on aspects of host behaviour, reproduction, fitness and degrees of reproductive isolation. This potential problem has already been underlined by work investigating the incidence of *Wolbachia* infections in stocks of the model system *Drosophila melanogaster*. Here we survey a range of lab stocks of further commonly used model arthropods, focussing especially on the flour beetles *Tribolium castaneum* and *T. confusum*, the cowpea weevil *Callosobruchus maculatus* and related species (Coleoptera: Tenebrionidae and Bruchidae). These species are widespread stored product pests so knowledge of infections with symbionts further has potential use in informing biocontrol measures. Beetles were assessed for infection with three known microbial reproductive parasites: *Wolbachia*, *Rickettsia*, *Spiroplasma*. Infections with some of these microbes were found in some of the lab stocks studied, although overall infections were relatively rare. The consequences of finding infections in these or other species and the type of previous studies likely to be affected most are discussed.

Key words: Coleoptera, reproductive isolation, reproductive parasite, sexual conflict, sexual selection, *Wolbachia*

Introduction

Manipulative reproductive parasites are known to be extremely common in arthropods. It has recently been estimated that one of these parasites, *Wolbachia*, infects an astonishing 66% of insect species (Hilgenboecker et al., 2008). Considering that insects constitute 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).

Wolbachia has also been shown to cause cytoplasmic incompatibility (CI) altering reproduction in a range of ways (Yen & Barr, 1971; Werren, 1997; Stouthamer et al., 1999; Duron et al., 2008a). CI may greatly reduce fertility and even cause sterility, with effects being either unidirectional (i.e. between infected and uninfected individuals), or bidirectional (i.e. between individuals infected with different *Wolbachia* strains). Such incompatibilities, especially when bidirectional, could limit gene flow amongst populations of a species and can be proposed to be influential in longer term evolutionary processes such as the development of reproductive isolation and, ultimately, speciation (Wade & Stevens, 1985; Breeuwer & Werren, 1990; Telschow et al., 2005). Finally, general effects on reproduction and fitness have also been documented (see Table 1 for an overview). These effects need not be negative, indeed *Wolbachia* infections are shown to increase resistance to particular viruses in *Drosophila* (Hedges et al., 2008, Osborne *et al.* 2012) and *Aedes aegypti* (Bian et al., 2010).

It has been suggested that the finding of the intracellular bacterium *Wolbachia* in ca. 30% of commonly used *Drosophila* stocks (housed at the Bloomington *Drosophila* Stock Center) might call into question the results of many evolutionary studies (Clark et al., 2005). The widespread occurrence of *Wolbachia* in such a ubiquitously used model organism is potentially alarming because it raises the possibility that differences in reproductive and/or fitness traits or compatibilities between populations might have a microbial basis rather than solely be caused by other proposed mechanisms.

It should be noted that the situation is more complicated than merely considering whether or not populations harbour *Wolbachia* (or any other individual reproductive parasite). Seemingly ‘uninfected’ stocks could well harbour other bacteria that can similarly affect their hosts (e.g. *Cardinium*, *Flavobacteria*, *Rickettsia*, *Spiroplasma*, *Arsenophonus*). A survey of stocks of different *Drosophila* species indeed finds that several species also harbour *Spiroplasma* (Tucson *Drosophila* Species Stock Center:

Mateos et al., 2006). Similarly extensive surveys have assessed infections with various reproductive parasites in further dipteran species belonging to the superfamily Muscoidea (ca. 70 species: Martin et al., 2012), and the Dolichopodidae and other Empidoidea (ca. 240 species: Martin et al., 2013a,b). Although *Wolbachia* infected flies more commonly, infections with *Spiroplasma*, *Rickettsia* and *Cardinium* were also found (Martin et al., 2012, 2013a,b). There is extensive evidence for both *Wolbachia* and *Spiroplasma* causing differences in host reproduction, including in Diptera (Duron et al., 2008a). Nevertheless, it is unclear how problematic the widespread infections in *Drosophila* stocks (Clark et al., 2005) actually are to the evolutionary studies carried out on them. How robust are conclusions drawn from previous experiments where the bacterium might – or might not – have been present? Here we review the type of experiments or traits under study that are likely to be most susceptible.

Considering how widespread such bacterial endosymbionts are among arthropods (Goodacre et al., 2006; Duron et al., 2008a; Hilgenboecker et al., 2008), and that *Wolbachia* is not the only microbe known to have such effects, we include in our study data from a range of model systems where we establish the presence of *Wolbachia* and of other microbes that are similarly implicated in altering the biology of their hosts. Conceivably, the presence of such parasites will be most relevant in model systems used extensively for studies on reproduction. Beyond *Drosophila melanogaster*, other lab organisms, which have (among other things) been used frequently for investigating reproductive biology, are beetles belonging to the genus *Tribolium* (Tenebrionidae). The red flour beetle *T. castaneum* is a widespread pest and has become a major model system for the study of pre- and postcopulatory sexual selection (Fedina & Lewis, 2008; Pai & Bernasconi, 2008; Michalczyk et al., 2010; Sbilordo et al., 2011; Grazer & Martin, 2012), and sexual conflict (Michalczyk et al., 2011a). This promiscuous species has also been used to assess the causes and consequences of polyandry, with recent examples focussing

on the roles played by inbreeding (Michalczyk et al., 2011b) and environmental change (Grazer & Martin, 2012). *T. castaneum* is also an important model in the study of host-parasite conflicts and immunity (e.g. Blaser & Schmid-Hempel, 2005; Zou et al., 2007; Wegner et al., 2008, 2009; Bérénos et al., 2009; Kerstes et al., 2013; Hangartner et al., 2013). Similar to *D. melanogaster*, one of the attractions of this system is the access to molecular tools such as the sequence of the entire genome of *T. castaneum* (Richards et al., 2008). Stocks of the related confused flour beetle *T. confusum* are already known to commonly harbour *Wolbachia*, with the microbe causing CI in this species (Fialho & Stevens, 1996). Interestingly, this CI-inducing *Wolbachia* strain is genetically indistinguishable (based upon sequences from four gene regions) from that infecting the congeneric species *T. madens* where it causes male killing (Fialho & Stevens, 2000).

Callosobruchus (Bruchidae) seed beetles are pests of stored legumes and can be easily reared in the lab. These species have also been the subject of intense study in the context of postcopulatory sexual selection (e.g. Wilson et al., 1997; Eady et al., 2004; Maklakov & Fricke, 2009), sexual conflict (Crudgington & Siva Jothy, 2000; Arnqvist et al., 2005; Rönn et al., 2007, 2011), and reproductive isolation (Fricke & Arnqvist, 2004), including targeted experimental evolution studies (e.g. Fricke & Arnqvist, 2007; Gay et al., 2009; Maklakov et al., 2009, 2010). *Callosobruchus* has also been the focus of detailed studies of the fitness consequences of ageing and inbreeding (Fox et al., 2004, 2006, 2011a,b; Bilde et al., 2009) including relationships with the environmental context (Messina & Fry, 2003; Fox et al., 2011b). Finally, many of the above representatives of the bruchid and tenebrionid beetles and closely related species are widespread pests of stored products. This adds an applied perspective, as *Wolbachia* has been discussed intensively as a potentially useful ally in the fight against pests and vectors of disease, for instance of mosquitos (see Laven, 1967; reviewed in Iturbe-Ormaetxe et al., 2011) or medflies (Zabalou et al., 2009). More generally, greater consideration of impacts of

symbionts on insect pests and vectors has been argued to be critical in assessing risks and effectiveness of biocontrol measures (Zindel et al., 2011).

In summary, the aims of the present study were two-fold: a) to survey a broad selection of commonly used laboratory strains of *Tribolium* and *Callosobruchus* beetles and related species for infection with three microbial reproductive parasites (*Rickettsia*, *Spiroplasma* and *Wolbachia*), and b) to assess consequences of finding such infections in these or other species and discuss the type of previous evolutionary study and data most likely to be at risk from the confounding effects of endosymbiont infections.

Materials and methods

Prior to testing, all the tenebrionid beetle stocks assayed in the present study had been maintained at large population sizes and housed on organic flour (with 10% brewer's yeast) in dark climate chambers at a constant 30 ° C (as standard for the stocks used, see Grazer & Martin, 2012). Although higher rearing temperatures are frequently used for tenebrionids, temperatures of above ca. 36 degrees are known to impact on endosymbiont infections, thus stocks that had been kept under these conditions in the past were avoided in our study (see e.g. Sakamoto et al., 2008). Bruchid beetle stocks were held in climate chambers at constant conditions of 27°C and 45% ($\pm$ 10%) relative humidity under a 12:12 h light-dark cycle. Beetles were held in 1L glass jars and maintained at large population sizes of 250-300 beetles per generation and provided with excess amounts of black-eyed beans (*Vigna unguiculata*).

Sample beetles for PCR testing were removed from their stock containers and subsequently kept in 70% ethanol until DNA extractions. DNA was extracted from abdominal tissue using QIAGEN DNEasy kits and eluted in 100 μ l distilled water. The 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 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' -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 µL containing 1 unit of Taq, 2.5 mM MgCl₂ ,
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

list of stocks tested for presence of *Rickettsia*, *Spiroplasma* & *Wolbachia* can be found in Table 2 and associated footnotes.

Results

Tenebrionidae

The results of our PCR survey for infections with the three endosymbionts are displayed in Table 2. Results confirm the presence, as expected, of *Wolbachia* in *T. confusum*, where it has previously been shown to cause CI (Wade & Stevens, 1985). In all *T. confusum* strains except HP70 both males and females were positive for *Wolbachia*. Previous studies have indicated that separate stocks may harbour identical (or at least compatible) *Wolbachia* strains (Fialho & Stevens, 1996). Preliminary crosses between infected and uninfected stocks appear to confirm this result (Martin, unpublished data). In contrast with *T. confusum*, individuals from the large number of *T. castaneum* strains tested were all apparently devoid of *Wolbachia* infections. Whereas in the former six out of eight stocks tested positive for *Wolbachia*, in contrast none of the ca. 40 *T. castaneum* strains tested appeared to be infected, although three of these were found to carry *Spiroplasma*, and one harboured *Rickettsia* (for details see Table 2). The closely related species *T. freemani* also appeared to be free of *Wolbachia* infection as was the single strain of *T. madens* tested in this survey. Others have shown that *T. madens* can be infected with *Wolbachia* strains genetically indistinguishable from that infecting *T. confusum* and that the bacterium distorts sex ratio by causing male-killing (Fialho and Stevens 2000). In accordance with the lack of infection in this study, no bias in sex ratio was apparent in the stock tested here (Martin, personal observation). Similarly, no *Wolbachia* infections were found in the remaining congeneric species (*T. anaphe*, *T. audax*, *T. brevicornis* or *T. destructor*) or any of the other tenebrionid species tested

(*Gnatocerus cornutus*, *Latheticus oryzae* or *Palorus ratzeburgii*) although we note that the number of samples tested for these species was very small (only a single individual in some cases) and thus our power to detect endosymbionts that are at anything less than 100% prevalence was low. Tests for *Rickettsia* and *Spiroplasma* detected neither of these types of bacteria in any of the individuals tested.

Bruchidae

Results are presented in Table 3. Testing 16 different populations of *Callosobruchus maculatus* and two related species *C. rhodesianus* and *C. analis* shows generally very low infection rates. *Spiroplasma* could not be detected from any of the samples while *Wolbachia* was only found in one individual out of four tested in *C. rhodesianus*. All three species *C. maculatus*, *C. rhodesianus* and *C. analis* show single infections with *Rickettsia*.

Discussion

Tests for endosymbiotic bacteria in the tenebrionid and bruchid beetles in this study appear to indicate that symbionts may be less common in these groups than in the insects assessed previously (Hilgenboeker et al., 2008). The overwhelming majority of currently available data are from studies on the interaction of insect hosts with *Wolbachia*, with far less being known about effects of other endosymbionts (examples in Table 1). Of the four classic phenotypes (CI, male-killing, feminization and parthenogenesis), all have been documented in a range of host species for *Wolbachia* and a few of these have also been shown to be caused by infections with other known endosymbionts. In *Tribolium* spp. specifically, research has focussed solely on *Wolbachia*, with evidence to indicate that this symbiont causes CI in *T. confusum* and male-killing in *T. madens* (Fialho & Stevens, 2000). Further impacts on non-reproductive traits are also possible as evidenced by recent

work suggesting a negative effect of *Rickettsia* infection on long-distance dispersal behaviour in a spider (Goodacre et al., 2009).

Precisely to what degree endosymbiont infections could confound results obtained from lab populations will depend on how the microbe affects the host. For example, if CI-causing bacteria remain undetected in particular insect stock populations, this could compromise studies involving inter-population crosses. Furthermore, if the stock populations in question are not uniformly infected, it could also explain differential reproductive successes across studies of single populations. Temporal changes in reproductive success of single populations might also occur if the natural rate of bacterial transmission from mother to offspring is altered under laboratory conditions, such that populations experience rapid changes in the frequency of endosymbiont infections after only a few generations in the lab. Such issues could be especially problematic when assessing reproductive isolation using postzygotic measures, as is often the case in studies directed towards understanding processes such as genetic isolation and speciation. Prezygotic measures could also be confounded if infection status affects mate preferences (see e.g. Markov et al., 2009) or the frequency of mating (Champion de Crespigny et al., 2006) (see also Table 1). It seems perhaps less likely that phenotypes involving sex ratio skew, such as parthenogenesis, feminization or male killing could ‘silently’ affect experimental populations. A strong bias towards females might appear likely to be picked up during routine work, although actual protocols used would need to be evaluated to assess possible risks of missing skewed sex ratios.

More general and less drastic negative (or positive) effects, for example on fitness are perhaps less likely to be an issue. Here it is unclear whether one could argue that patterns would be majorly influenced by undetected endosymbionts, unless populations used are not uniformly infected. Laboratory populations will also be, or have been, affected by a large range of other intrinsic and extrinsic factors. These remain for the

most part equally silent, and may for example include nematodes, mites, other pathogens or parasites, or selfish genetic elements such as *Medea* in *T. castaneum* (Lorenzen et al., 2008). In this respect, reproductive parasites are probably not truly a greater challenge than any other of these unknowns, which already have to be taken into account.

Artificial transfer experiment protocols exist for *Tribolium* beetles (Chang & Wade, 1996), potentially offering a controlled way to assess effects on existing (or novel) hosts experimentally. Indeed, the effects on reproduction of the various symbionts remain largely unresolved for many populations (or species). Reproductive parasites can specifically impact on reproductive traits (see Table 1), so beyond obvious involvement in conflict between host and symbiont, they can impinge on sexual conflict between males and females (see Martin & Gage, 2007). A promising and targeted means of illuminating the separate and combined action of these (interspecific and intraspecific) evolutionary conflicts would be to use a combined experimental evolution approach akin to previous experiments focusing on either sexual conflict (e.g. Martin & Hosken, 2003, 2004; Fricke & Arnqvist, 2007; Gay et al., 2009; Hosken et al., 2009; Maklakov et al., 2010; Michalczyk et al., 2011a) or host-parasite conflict (e.g. Bérénos et al., 2009). Findings of experimental evolution studies in *Tribolium* and *Callosobruchus* (e.g. Gay et al., 2009; Maklakov et al., 2009, 2010; Michalczyk et al., 2011a) coupled with detailed knowledge of reproduction in these study systems could provide a solid base for understanding interactions between hosts and their reproductive parasites.

One means of assessing symbiont effects has been to treat animals with antibiotics to cure them of their infections. However, treatment with this antibiotic also has the potential to influence other fitness traits and likely eliminates other known or unknown bacteria with unpredictable consequences. Furthermore, there are potentially other confounding effects, such as persistent effects on metabolism, after curing with Tetracycline (see e.g. Ballard & Melvin, 2007).

Infections with *Rickettsia* and *Spiroplasma* are found across a wide range of arthropods so were hence also tested for in this study in addition to *Wolbachia*. In fact, multiple infections within species or groups of species are not uncommon (e.g. Weeks et al., 2003; Goodacre et al., 2006). In this study we only found very few infected individuals and only one multiply infected female (*C. rhodesianus* infected with both *Wolbachia* and *Rickettsia*, in contrast with Kondo et al., 1999; see Table 3). More generally, though, further complications could arise if different infections interact with one another. Such inter-microbial interactions may be a promising area of future research (see e.g. Engelstädter et al., 2008).

Clearly evolutionary biologists need to be aware of the complex relationship between a study organism and its associated symbionts or parasites. Studies such as this or the large-scale work already undertaken on *Drosophila* (Clark et al., 2005, Mateos et al., 2006) and other Diptera (Martin et al., 2012) can only be informative. Researchers should be grateful rather than alarmed that leading lab ‘work-horses’ such as *Drosophila*, *Tribolium* or *Callosobruchus* are not impervious to the range of microbial diversity commonly found in the wild. For a start, the majority of arthropod species are likely to have evolved in contact with *Wolbachia*, so study organisms infected with this parasite are probably more representative of the situation in the wild. Moreover, this should really be seen as a valuable opportunity to address pressing questions in a burgeoning area of research, using the well-understood systems that model lab organisms such as *Tribolium* provide. Here one can draw not only upon a wealth of extensive and highly relevant information on host reproduction but also access the full array of genetic tools available for these species.

To conclude, we provide data on infections with three common reproductive parasites in stock populations of the popular model systems *T. castaneum* and *C. maculatus* and a range of related species. We confirm an emerging pattern where

Wolbachia infections are widespread in *T. confusum* stocks, yet the same types of bacteria (*i.e.* those that are sensitive to our detected methods) appear to be conspicuously absent in other Tenebrionidae assessed (see also Chang & Wade, 1996; Kageyama et al., 2010). Additionally, our results confirm a lack of *Wolbachia* infections in *C. maculatus* matching previous surveys (Kondo et al., 1999; Kageyama et al., 2010). In contrast, *Wolbachia* has previously been documented in *C. analis* and *C. chinensis* (Kageyama et al., 2010). However, symbionts other than *Wolbachia* were not assessed in previous surveys where tenebrionid or bruchid host species were included (e.g. Kageyama et al., 2010). Here, *C. maculatus* is found to harbour infections with *Rickettsia*, illustrating the point that assessing several symbionts is worthwhile (this also holds for *T. castaneum*, see Table 2).

It is important to emphasise that our failure to detect bacterial DNA in particular species/stocks included in this study does not imply that these are necessarily endosymbiont free. It only implies that the individuals that we tested do not carry bacterial strains that we can detect and we note the number of individuals that we have tested in our study is small. Low prevalence of endosymbionts, such as male killers, (which may have a lower prevalence within a population than their CI-inducing counterparts) within a population or very low bacterial titres would make it less likely that they would be detected in our study. Furthermore, divergent bacterial strains can remain undiagnosed even if at high prevalence and/or high titre if they are not detected by our PCR methods (e.g. as demonstrated by Simões *et al.* 2011). The use of next generation sequencing technology to sequence all those bacteria found within in combination with more comprehensive sampling may be a useful step forward in the study of endosymbionts in model lab organisms, as has been applied for other invertebrate groups (e.g. Kautz *et al.* 2013.)

Overall, we suggest that the widespread distribution of reproductive parasites in lab stocks is not by itself a basis for universal concern. Clearly, however, earlier interpretations should always be open to additional scrutiny or re-evaluation if necessary, i.e. if stocks are infected. As a case in point, we find that the *T. castaneum* source population used in several recent studies (Morrow et al., 2003; Michalczyk et al., 2010, 2011a,b; Sbilordo et al., 2011; Grazer & Martin, 2012; Hangartner et al., 2013) is free of infection with the symbionts assessed. We further propose that valuable new insights could be gained by considering new data on bacterial infections including all known reproductive parasites in further hosts. This may be particularly useful in model systems for sexual selection and related themes such as the genera *Tribolium* and *Callosobruchus* where extensive knowledge of reproduction is already available. Finally, more detailed knowledge accrued concerning infections can help build strong foundations for mounting biocontrol measures against target taxa (see e.g. Xi et al., 2005).

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Disclosure

The authors declare that they have no conflict of interests.

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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)
<u>Reproductive isolation</u>		
Prezygotic (mate choice)	Infection status affects assortative mating	<i>Wolbachia: 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: Tribolium confusum</i> (Wade and Stevens 1985)
<u>Reproductive traits</u>		
FEMALES		
Female fecundity	Infection associated with decrease	<i>Wolbachia: Tribolium confusum</i> (Wade and Chang 1995)
Oviposition behaviour	Infected females aggregate offspring (to promote matings between siblings?)	<i>Wolbachia: Tetranychus urticae</i> (Vala et al. 2004)
MALES		
Male fertility	Infection associated with increase	<i>Wolbachia: Tribolium confusum</i> (Wade and Chang 1995)
Sperm competitive ability	Infection associated with reduced sperm competition success	<i>Wolbachia: Drosophila simulans</i> (Champion de Crespigny and Wedell, 2006)
Male mating rate	Infected males mate at higher rates than uninfected counterparts	<i>Wolbachia: 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: Encarsia formosa</i> (Zchori-Fein et al. 1992)

Skewed sex ratios	Decreased numbers of males in a population could be caused by feminization ...	<i>Wolbachia: Ostrinia furnacalis</i> (Kageyama et al. 2002)
	... or could be due to male-killing	<i>Flavobacteria: Adonia variegata</i> (Hurst et al. 1999)
<u>Non-reproductive traits</u>		
Dispersal behaviour	Infected females are less likely to disperse	<i>Rickettsia: Erigone atra</i> (Goodacre et al. 2009)
Survival	Infection increases longevity	<i>Wolbachia: Drosophila melanogaster</i> (Fry and Rand 2002)
Thermotolerance	Infection increases tolerance to heat shock	<i>Rickettsia: Bemisia tabaci</i> (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 (Ningbo, 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 and 10 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
<i>Gnatocerus cornutus</i>	(USDA)		-	-	-
<i>Latheticus oryzae</i>	(USDA)		-	-	-
<i>Palorus ratzeburgii</i>	(USDA)		-	-	-
<i>Tribolium anaphe</i> *	(CSL)		-	-	-
<i>Tribolium audax</i> *	(CSL)		-	-	-
<i>Tribolium brevicornis</i>	(USDA)		-	-	-
<i>Tribolium destructor</i> *	(CSL)		-	-	-
<i>Tribolium castaneum</i>	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	-	-	-	-
<i>Tribolium confusum</i>	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	+	-	-
<i>Tribolium freemani</i>	(USDA)	Japan	-	-	-
<i>Tribolium madens</i>	(USDA)	Kansas	-	-	-

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in 3 bruchid beetle species. *Callosbruchus* beetle stocks were screened for infection with the 3 endosymbionts *Wolbachia*, *Rickettsia* and *Spiroplasma* using PCR: ‘+’ indicates positive infection status and ‘-’ no infection. N=1 individual per sample. Location names (where known) indicate where beetles were sampled.

	Location	Sex	<i>Wolbachia</i>	<i>Rickettsia</i>	<i>Spiroplasma</i>
<i>C. maculatus</i>	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	-	-	-
<i>C. rhodesianus</i>	Swaziland	M	-	-	-
	Swaziland	F	-	-	-
	Swaziland	F	+	+	-
	Swaziland	M	-	-	-
<i>C. analis</i>		F	-	+	-
		M	-	-	-
		F	-	-	-
		F	-	-	-

Notes: The Brazil and South India strains were split and held in different laboratories (by G. Keeney (USA) and R. Smith (Leicester)) - details of their history can be found in Fricke and Arnqvist 2004. The origins of the Nigeria mix and Poly B 4 strains can be found in Fricke and Arnqvist (2007) (for other stocks and further details see: Giga & Smith, 1991, Rönn et al., 2007, 2011, Rankin & Arnqvist, 2008).