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Infections with *Wolbachia* and *Spiroplasma* in the Scathophagidae and other Muscoidea

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ABSTRACT

The microbes *Wolbachia* and *Spiroplasma* are common reproductive parasites of arthropods and may strongly influence reproduction of infected hosts and also impact on reproductive isolation. Such infections could hence influence results of many studies assessing reproductive behaviour and fitness of possible hosts, as well as reproductive isolation. Previous work indicates that infections with the microbes *Wolbachia* and *Spiroplasma* are common in the Drosophilidae. However, extensive and targeted surveys of other Dipteran families are lacking. Here we survey the yellow dung fly *Scathophaga stercoraria* and a range of other species from the Muscoidea (families Scathophagidae, Anthomyiidae, Fanniidae and Muscidae) collected in the field or obtained from museum collections for infection with the widespread reproductive parasites *Wolbachia* and *Spiroplasma*. Both have been shown to be heritable symbionts and affect reproduction in other Diptera. *S. stercoraria* is a very important model for the study of sexual selection, and in particular of postcopulatory processes, as it has played a major role in the history of research on sperm competition and cryptic female choice. Infections with *Wolbachia* were found to be widespread across the Muscoidea, whereas infections with *Spiroplasma* were rarer. We discuss the consequences of these findings and directions for future research on the impact of reproductive parasites on host reproduction in the Scathophagidae.

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1. Introduction

Microbes with the potential to act as manipulative reproductive parasites are extremely common in arthropods. A recent estimate for one of these parasites, *Wolbachia pipientis*, suggests that it may infect a staggering 66% of insects worldwide (Hilgenboecker et al., 2008). Insects represent ca. 75% of global biodiversity (Grimaldi and Engel, 2005), so this single parasite species infects about 50% of all extant species. Beyond being highly successful in insects, *Wolbachia* also infects many other taxa, including spiders (Goodacre et al., 2006), mites (Breeuwer, 1997), ticks (Noda et al., 1997), crustaceans (Cordaux et al., 2001) and filarial nematodes (Taylor and Hoerauf, 1999). *Wolbachia* was discovered by Hertig and Wolbach in 1924 and since then scientists have become aware of its potential impacts on host biology (Yen and Barr, 1971).

We now also know of a range of other microbes with comparable effects (reviewed in Duron et al., 2008a): e.g. *Arsenophonus* (Gherna et al., 1991), *Cardinium* (Zchori-Fein et al., 2004), *Flavobacterium* sp. (Hurst et al., 1997, 1999a), *Rickettsia* (Werren et al., 1994) or *Spiroplasma* sp. (Hackett et al., 1986; Hurst et al., 1999b). Taken together with *Wolbachia*, these symbionts infect for instance ca. 50% of spiders (Duron et al., 2008a,b; Goodacre et al., 2006; Martin and Goodacre, 2009). Overall, reproductive parasites undoubtedly affect a bewildering number of arthropods. Other microbes have generally been examined much less extensively than *Wolbachia*, and there are also probably many undescribed microbes with similar effects.

It is known that maternally inherited microbes, such as *Wolbachia*, may manipulate host reproduction to promote their own transmission (Engelstädter and Hurst, 2009). The main route of transmission for these microbes is vertical (i.e. from mother to offspring), so for reproductive parasites it is advantageous to favour a female-biased sex ratio (Duron et al., 2008a; Majerus and Majerus, 2010; Stouthamer et al., 1999). Such a distorted sex ratio can be achieved via different means: male-killing, feminization of male embryos or by inducing thelytokous parthenogenesis. *Wolbachia* can further cause cytoplasmic incompatibility (CI), which is associated with a range of reproductive alterations (Duron et al., 2008a; Stouthamer et al., 1999; Yen and Barr, 1971). CI can cause reductions in fertility up to full sterility, with effects being either unidirectional (i.e. between infected and uninfected individuals), or bidirectional (i.e. between individuals infected with different *Wolbachia* strains). Incompatibilities of this kind may limit gene flow, especially if bidirectional, and thus exert an influence on

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reproductive barriers (e.g. Hurst and Schilthuizen, 1998; Wade and Stevens, 1985). Other effects on reproduction, behaviour and fitness have also been documented (see e.g. Champion de Crespigny and Wedell, 2006; Champion de Crespigny et al., 2006; Goodacre et al., 2009). In fact, effects need not always be negative, as some infections afford increased resistance to viruses (Hedges et al., 2008).

Wolbachia has recently been found to infect approximately 30% of *Drosophila* stocks at the Bloomington *Drosophila* Stock Center (Clark et al., 2005). The presence of *Wolbachia* in this extensively used model system raises the possibility that documented differences in reproductive traits or compatibility between populations might actually relate to infections with reproductive parasites (see Clark et al., 2005). As outlined above, though, seemingly 'uninfected' stocks might actually harbour other microbes with potentially similar effects on their hosts (e.g. *Cardinium*, *Flavobacteria*, *Rickettsia*, *Spiroplasma*). Indeed, one recent survey of stocks from a range of different *Drosophila* species at the Tucson *Drosophila* Species Stock Center finds that some species are infected with *Spiroplasma* (Mateos et al., 2006). Considering precisely how extraordinarily common such microbial endosymbionts are among arthropods, it would be useful to have comprehensive data from other well-studied systems.

The presence of reproductive parasites is highly relevant for model systems used in studies on reproduction such as *Drosophila*. Another classic Dipteran model organism for investigations of pre- and postcopulatory sexual selection is the yellow dung fly *Scathophaga stercoraria* L. (Diptera: Scathophagidae) (see Sbilordo et al., 2010). Since early pioneering work on sperm competition by Geoff Parker in the 1970s (Parker, 1970a,b), the species has been targeted in numerous investigations of pre- and postcopulatory sexual selection including cryptic female choice (Bussière et al., 2009; Demont et al., 2011; Pitnick et al., 2009; Sbilordo et al., 2009; Simmons, 2001; Ward, 2000, 2007) and male–female coevolution (Minder et al., 2005). *S. stercoraria* has further been the focus of research on sexual conflict, including experimental evolution incorporating monogamous versus polyandrous selection regimes (Hosken et al., 2001; Hosken and Ward, 2001; Martin et al., 2004).

Another research area where infections with reproductive parasites could generate confounds is the study of reproductive isolation. CI seems most relevant here as this phenotype could lead to variation in reproductive success if laboratory populations under study are not uniformly infected. CI could also underly outcomes of crosses between populations if assessed via postzygotic measures. Prezygotic measures are also not immune in cases were infection status shapes mate preferences (e.g. Markov et al., 2009). Overall, reproductive parasites could have profound effects on a range of reproductive traits, and have consequences for the study of sexual selection, sexual conflict and speciation. In *S. stercoraria*, previous research has also involved assessment of interpopulation crosses (e.g. Hosken et al., 2002), so data on possible infections with microbes such as *Wolbachia* would be welcome.

Here we specifically survey *S. stercoraria*, and other species from the Dipteran superfamily Muscoidea (Scathophagidae, Anthomyiidae, Fanniidae and Muscidae) for infection with two common reproductive parasites, *Wolbachia* and *Spiroplasma*, mirroring recent efforts on the genus *Drosophila* (Clark et al., 2005; Mateos et al., 2006). Both these symbionts are known to infect Diptera and manipulate host reproduction in flies, e.g., *Wolbachia* causes CI in *Culex pipiens* (Yen and Barr, 1971) and *Spiroplasma* male-killing in *Drosophila willistoni* (Hackett et al., 1986). *Wolbachia* has also been shown to increase male mating rate (Champion de Crespigny et al., 2006) and decrease sperm competitive ability in *Drosophila* (Champion de Crespigny and Wedell, 2006).

2. Materials and methods

2.1. Samples, DNA extraction, amplification, sequencing

We made use of extensive DNA samples from previous studies (Bernasconi et al., 2000, 2001, 2010; Kutty et al., 2007) to screen *S. stercoraria*, a range of representatives of the Scathophagidae, and species from the muscoid families Anthomyiidae, Fanniidae and Muscidae for infection with *Wolbachia* and *Spiroplasma* via PCR and direct sequencing. DNA was extracted from whole fly specimens using DNeasy Tissue kits (Qiagen AG, Hombrechtikon, Switzerland) according to manufacturer's instructions. Whole specimens were triturated mechanically in microtubes using a TissueLyser (Mixer Mill MM 300, Qiagen AG, Hombrechtikon, Switzerland). Following digestion with Proteinase K (20 μg/ml), samples were applied to the columns for DNA absorption and washing. Finally, DNA was eluted in 200 μl of the buffer from the kit and stored at −20 °C. All the extracted fly specimens are deposited at the Zoological Museum, Institute of Evolutionary Biology and Environmental Studies, University of Zurich. Standard PCR reactions were performed with 2 μl of the extracted DNA as template, 1 μl of each primer (10 μM), 12.5 μl Master Mix (250 units, HotStarTaq Master Mix Kit, Qiagen AG, Hombrechtikon, Switzerland), 8.5 μl distilled H_2O, for a total volume of 25 μl (manufacturer's buffer). The following primers (Microsynth GmbH, Balgach, Switzerland) were used: (i) *Wolbachia* (*wsp* gene) WSP-F: TGGTCCAATAAGTGATGAAGAAACTAGCTA and WSP-R: AAAAATTAAACGCTACTCCAGCTTCTGCAC (Jeyaprakash and Hoy, 2000); (ii) *Spiroplasma* (intergenic ribosomal spacer and adjacent regions between the 3′-end of 16S and the 5′-end of the 23S), SP-ITS-J04: GCCAGAAGTCAGTGTCCTAACCG and SP-ITS-N55: ATTCCAAGGCATCCACCATACG (see Majerus et al., 1999). For both microbes, the PCR reaction mixtures were subjected to 10 min DNA denaturation at 95 °C, 50 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 20 s, and elongation at 72 °C for 30 s. Elongation was completed by a further 7 min step at 72 °C. The PCR reactions were performed in a DNA Thermal Cycler (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland). Purification of the PCR products for direct sequencing was performed by adding 0.5 μl (1 U/μl) Shrimp Alkaline Phosphatase (Promega AG, Wallisellen, Switzerland), 0.25 μl (20 U/μl) Exonuclease I (New England Biolabs (Bioconcept), Allschwil, Switzerland), and 24.25 μl distilled H_2O (ratio of PCR product and ExoSap-mix 1:1) to each PCR product. The ExoSAP protocol consisted of 45 min incubation at 37 °C and 15 min deactivation at 80 °C. Cycle sequencing reactions were performed in total volumes of 10 μl using an ABI Prism Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland) on an ABI 3730 DNA Analyser (Perkin-Elmer Applied Biosystems). Negative controls were used at each step of the amplification and sequence procedures. These negative controls consisted of microtubes/positions in the reaction plates containing all the necessary reagents except that the extracted genomic DNA or the purified PCR product to be amplified or sequenced was substituted with distilled H_2O. Positive controls, consisting of extracted genomic DNA of samples infected with the microorganisms under study (for the PCR) and in purified PCR products (for the direct sequence) were used at each step of the lab procedure as well.

2.2. DNA sequence analyses

Sequences for both *Wolbachia* and *Spiroplasma* were handled and stored with the Lasergene program Editseq (DNAstar Inc., Madison, WI USA). Alignment of all gene sequences was performed using the Clustal W method as implemented in Megalign (DNAstar Inc.) with default multiple alignment parameters ("gap penalty = 15"; "gap length penalty = 6.66"; "delay divergent sqs

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