Cover and lay-out: Bert Inghelbrecht

Vanthournout, B. 2012. Sex ratio distortion in the male dimorphic dwarf spider *Oedothorax gibbosus*: mechanisms and the role of endosymbiont bacteria, PhD Thesis, Ghent University.

ISBN:

SEX RATIO DISTORTION IN THE MALE DIMORPHIC DWARF SPIDER *OEDOTHORAX GIBBOSUS*: MECHANISMS AND THE ROLE OF ENDOSYMBIONT BACTERIA

BRAM VANTHOURNOUT

GHENT UNIVERSITY, FACULTY OF SCIENCES

Thesis submitted in fulfilment of the requirements for
the degree of Doctor (PhD) in Sciences, Biology

PROEFSCHRIFT VOORGELEGD TOT HET BEHALEN VAN DE GRAAD VAN DOCTOR IN DE WETENSCHAPPEN, BIOLOGIE

SUPERVISOR

Prof. Dr. Frederik Hendrickx (KBIN-IRSNB, Ghent University, Belgium)

READING COMMITTEE

Prof. Dr. Thierry Backeljau (KBIN-IRSNB, Belgium)
Prof. Dr. Trine Bilde (Aarhus University, Denmark)
Prof. Dr. Ellen Decaestecker (Catholic University of Leuven, Belgium)
Dr. Eduardo De La Peña (Ghent University, Belgium)

EXAMINATION COMMITTEE

Prof. Dr. Frederik Hendrickx (Supervisor, KBIN-IRSNB, Ghent University, Belgium)
Prof. Dr. Dries Bonte (Chairman, Ghent University, Belgium)
Dr. Frederik Leliaert (Ghent University, Belgium)
Prof. Dr. Thierry Backeljau (KBIN-IRSNB, Belgium)
Prof. Dr. Trine Bilde (Aarhus University, Denmark)
Prof. Dr. Ellen Decaestecker (Catholic University of Leuven, Belgium)
Dr. Eduardo De La Peña (Ghent University, Belgium)

CONTENTS

“Wat was het nu ook al weer Dat ik wou doen Dat ik wou bewijzen”

from ‘Veronica komt naar je toe ‘
Luc Devos, Gorki

GENERAL INTRODUCTION

FISHER

Although it is considered that Fisher (1930) first provided a formal explanation for the ubiquity of equal amounts of males and females in eukaryotic species with two sexes, the very first argumentation dates back to Darwin's second book "The Descent of Man and Selection in Relation to Sex" (1871). Here, he touches upon the idea of an equal sex ratio being selected for in biased populations due to a higher fitness at mating of the rarer sex. However, in the second edition of the book, he abandons this and leaves it to future generations to solve. A mathematical argument was subsequently provided by Düsing (1883, 1884). Nonetheless, the work of Fisher is considered to be path breaking in sex ratio theory (Charnov, 1982). He argues that as each offspring has one mother and one father, the total reproductive value of males should be equal to that of females (Fisher, 1930). Consider a sex ratio biased population in which it is equally costly to produce daughters and sons. The per capita reproductive value will be higher for the underrepresented sex and this will select for genotypes expressing this rarer sex, which increases in the population and ultimately leads to an equal sex ratio. A numerical example is given in table 1. In a male biased population, males will obtain on average less matings than females, rendering the average reproductive value of females higher than that of males and favours the production of the female sex. The same argumentation holds for a female biased population in which males will experience a higher average reproductive value as more matings are obtained than females, favouring the production of the male sex. Only in an unbiased population males and females have an equal average reproductive value and consequently the production of equal amounts of males and females is favoured.

Population sex ratio	Number in population	Reproductive value per individual	Selection favors individuals who produce an
Male biased			
Males	200	100/200 = 0.5	Female biased sex ratio
Females	100	1	
Female biased			
Males	100	200/100 = 2	Male biased sex ratio
Females	200	1	
Unbiased			
Males	150	150/150 = 1	Unbiased sex ratio
Females	150	1	

Table 1. A numerical illustration of Fisher's theory. The relative reproductive values of male and female offspring are given for situations where the population sex ratio is biased towards males or females or is unbiased. The mean reproductive value of females is assumed to be 1.0, and the mean reproductive value of males is given by the average number of matings they will obtain multiplied by the mean reproductive values of females, which is given by (number of females/number of males) x 1.0 (adopted from West, 2009).

The implications of Fisher's model are important for two reasons (West, 2009). First, it illustrates the negative frequency dependence of sex ratio selection, that is, a bias towards one sex causes a rise in the reproductive value of the other sex. Secondly, it provides a basic theory for sex allocation; if sex ratio bias is detected, this suggests that other factors play a role causing this deviation from an even sex ratio. It should be emphasized that predictions of this model also allow to predict biased production of male and female offspring. If, for example, males are twice as costly to produce than females, the sex ratio should be biased towards females (approximately twice as much females than males) as parents are assumed to invest equal amounts of resources into male and female offspring (West, 2009). Sex ratio biases could therefore be explained in terms of relaxed assumptions of the general Fisherian model (Hamilton, 1967, West, 2009). We discuss some of these relaxed assumptions (competition/cooperation between individuals and non-Mendelian segregation of alleles determining sex allocation) below.

LOCAL RESOURCE COMPETITION / ENHANCEMENT

In the absence of competition and/or cooperation between individuals the production of equal amounts of males and females is predicted. However, the occurrence of such interactions has been suggested to explain the existence of biased sex ratios. Two categories can be distinguished, local resource enhancement (LRE) and local resource competition (LRC).

Local resource enhancement occurs when the production of a certain sex enhances the fitness of related individuals, therefore favouring the biased production towards this particular sex (Trivers and Willard, 1973, Taylor, 1981, West, 2009). In the Seychelles warbler, females regularly remain in the nest to serve as helpers of the breeding couple by aiding in territory defense and feeding of the young. This suggests that sex ratio selection should favour the production of the helping sex, i.e. females. Indeed, female biased sex ratios are observed in territories that benefit from the presence of helping females (Komdeur, 1996, Komdeur *et al.*, 1997, West, 2009).

In local resource competition production of a certain sex causes increased levels of competition between relatives for resources, this favours the production of the sex resulting in lower levels of competition (Bulmer and Taylor, 1980, Taylor and Bulmer, 1980, Taylor, 1981). A special case of LRC is local mate competition (LMC) (Hamilton, 1967). If related males strongly compete for access to females, a female biased sex ratio is favoured. Bark beetles of the weevil subfamily Scolytinae can be divided into two groups according to their time of mating, one group mates before dispersal of their larval host and the other at the new host, after dispersal from their native host. If mating occurs before dispersal, LMC among male relatives is considered to be high and hence female biased sex ratios are expected. In the case mating occurs after dispersal, unrelated males compete against each other, decreasing LMC and equal sex ratios are expected. In line with these predictions, highly female biased sex ratios are found in the pre-dispersal mating group and even sex ratios in the group with mating after dispersal (Hamilton, 1967, Kirkendall, 1993, Jordal *et al.*, 2002). More experimental evidence has recently been obtained in a mite species as female biased sex ratios were observed in the presence of high levels of local mate competition (Macke *et al.*, 2011).

SELFISH GENETIC ELEMENTS

A key insight into the existence and actions of selfish genetic elements comes from the understanding that selection can work on individual genes rather than on the individual itself (Gershenson, 1928, Östergren, 1945, Dawkins, 1976, Doolittle and Sapienza, 1980, Orgel and Crick, 1980). Hence, if these elements alter their transmission to subsequent generations, by distorting the Mendelian inheritance pattern, their fitness will increase (reviewed in Werren *et al*., 1988, Lyttle, 1991, Werren, 2011). We focus here on sex ratio distorting elements, causing a marked sex ratio bias in their host, as a result increasing their transmission into the next generation. This sex ratio skew is in strong contrast with the equal sex ratio favoured by the host according to the theory of Fisher (1930), therefore resulting in the occurrence of genetic conflict (see further). They can be classified according to their location in the host, nuclear or cytoplasmic (Stouthamer *et al*., 2002, West, 2009). An overview of the effects of the different types of selfish genetic elements is given in table 2.

NUCLEAR GENES

SEX-CHROMOSOME MEIOTIC DRIVE GENES

Meiotic drive is the non-Mendelian inheritance of alleles on homologous chromosomes or heteromorphic chromosomes (sex-chromosomes), resulting in the unequal transmission of both alleles in the offspring (Lyttle, 1991, Jaenike, 2001). In the absence of meiotic drive, individuals of the heterogametic sex produce equal amounts of male and female determining gametes resulting, after fertilization, in an equal production of male and female offspring.
However, sex-chromosome meiotic drive results in the presence of the driving chromosome in more than half of the gametes produced which causes the production of biased amounts of male and females. This phenomenon was first discovered in *Drosophila obscura* as a female biased sex ratio distortion was observed, inherited only through males (Gershenson, 1928). In this species males are the heterogametic sex as they produce X- and Y-chromosome bearing sperm. It was observed that the female bias correlated with a lower production of Y-sperm, and as the sex ratio bias was inherited only through male offspring, a factor located on the X-chromosome was thought to be the causative agent. Subsequent work on the mechanism of sex-chromosome drive revealed that a driving gene present on the X-chromosome causes aberrations in the meiotic development of sperm cells containing the Y-chromosome (Montchamp-Moreau and Joly, 1997, McKee, 1998, Cazemajor *et al*., 2000, Wilkinson and Sanchez, 2001). X-drive has been primarily documented from a number Diptera species such as *Drosophila* species, sciarid flies and stalk-eyed flies (overview in West 2009) and only scarcely in other taxa such as vertebrates (Fregda *et al*., 1976, Fregda *et al*., 1977) and plants (Taylor, 1999). In contrast with the female biased sex ratio caused by X-drive, a male biased sex ratio resulting from Y-drive has been far less observed (Hickey and Craig, 1966). It is not entirely clear why Diptera species seem particularly prone to exhibit sex-chromosome drive but it can be argued that drive should arise in species where males are the heterogametic sex. The loss of half of the produced gametes would be less detrimental in males than in females as sperm is normally produced in excess (Majerus, 2003).

SUPERNUMERARY CHROMOSOMES

Supernumerary (B) chromosomes are not essential for survival and function of the organism and can be present in multiple copies per individual, additional to the standard complement of chromosomes (A)(Hurst and Werren, 2001, West, 2009, Camacho *et al*., 2011). Although in general few sex ratio effects are documented, a particularly well studied system is the supernumerary chromosomal PSR (psr) element present in *Nasonia vitripennis* (Hymenoptera). In this species with haplodiploid sex determination, females normally develop from fertilized eggs while males originate from unfertilized eggs. However, the PSR element, which is only present in males, causes after fertilization of the egg a dense condensation of the entire paternal genome, except itself. This potential loss of the paternal genome renders fertilized eggs haploid which subsequently develop into PSR infected males (Werren *et al*., 1981, Nur *et al*., 1988, Werren and Stouthamer, 2003). The penetrance of this effect is very high as 99% of eggs develop as males. A male biased sex ratio is favoured from the perspective of the PSR element as it is passed on to all the sperm cells produced by a haploid male, while in diploid females it ends up in only half of the egg cells. It is regarded as one of the most ultimate selfish genetic elements as it can not only influence the host sex ratio towards the production of all males, it actively destroys the rest of the male genome, depleting the genetic contribution of the male to the next generation. A second PSR element has been detected in the parasitoid wasp *Trichogramma kaykai* (Stouthamer *et al*., 2001) and a PSR like mechanism is observed in the parasitoid wasp *Encarsia pergandiella* (Hunter *et al*., 1993). In other species effects of supernumerary chromosomes on the sex ratio are expected as they are found in a higher frequency in males than in females (Imai, 1974, Morgan-Richards, 2000, de Brito Portela-Castro *et al*., 2001) or in the reverse situation of a higher frequency in females (Vicente *et al*., 1996, Neo *et al*., 2000).
More convincingly, a correlation with the occurrence of a sex ratio distortion has been found in Anostraca and cichlid fishes (Beladjal *et al*., 2002, Yoshida *et al*., 2011).

Selfish element	Offspring sex ratio	Inheritance pattern of the sex ratio bias
Meiotic drive genes		
X-chromosome	Female biased sex ratio	Paternal
Y-chromosome	Male biased sex ratio	Paternal
B-chromosomes	Male biased sex ratio	Paternal
Endosymbiont bacteria	Female biased sex ratio	Maternal

Table 2. Overview of the effects of different types of selfish elements on the offspring sex ratio produced by females harbouring this element and indication of the inheritance pattern of the sex ratio bias.

ENDOSYMBIONT BACTERIA

Symbiosis is defined as the living together of dissimilar organisms (de Bary, 1879). Interactions between these organisms can range from a mutualistic, commensal to a parasitic nature.

The most commonly known cases of symbiosis within the arthropod order are the protist and bacterial communities present in the digestive system of termites and the endosymbiont bacteria in aphids. The degradation of lignocellulose, allowing termites to feed on a variety of plant fibers is ascertained by the action of digestive enzymes produced by a large number of protist and bacterial species present in the hindgut (Martin, 1991, Ohkuma, 2008, Kudo, 2009). In aphids, bacterial endosymbionts of the genus *Buchnera* are present in specialized cells, termed bacteriocytes, which provide essential amino acid nutritioning (Douglas, 1998). These interactions can clearly be regarded as beneficial as endosymbiont presence is necessary for survival of the host. However, negative interactions can also be found. One such example is infection with endosymbiont bacteria that distort the sex ratio of their host. These bacteria are considered to be obligatorily intracellular (but see further *Spiroplasma* and *Arsenophonus*) and cannot survive outside the host. Therefore, as they are almost exclusively maternally inherited through the cytoplasm of the egg cell, males are considered evolutionary dead ends. Because of this maternal inheritance pattern biasing the sex ratio of the host towards females is favoured from the perspective of the endosymbiont. As this contradicts with an even sex ratio favoured by the host, these endosymbionts can be regarded as reproductive parasites (Werren and O'Neill, 1997, Bandi *et al.*, 2001). Several strategies have evolved that result in a higher proportion of infected females. These are male-killing, feminization, parthenogenesis induction and the occurrence of cytoplasmic incompatibility (Werren, 1997, Stouthamer *et al.*, 1999, Charlat *et al.*, 2003, Werren *et al.*, 2008, Engelstadter and Hurst, 2009).

MALE-KILLING

A male-killing phenotype results from the death of male embryos, early in the embryonic development, while female embryos have a normal development resulting in a female-biased sex ratio. Due to male mortality, infected females typically produce half the number of offspring compared to uninfected females. Although killing of males does not directly result in higher numbers of infected females, indirect benefits are suspected such as reduced competition between female offspring, reduced levels of inbreeding and nutritional advantages by consuming dead brothers (Hurst and Majerus, 1993, Majerus and Hurst, 1997, Charlat *et al.*, 2003, Elnagdy *et al.*, 2011). Male-killing endosymbionts have been found in several arthropod orders (Hurst and Jiggins, 2000) such as Diptera (Williamson and Poulson, 1979), Lepidoptera (Jiggins *et al.*, 2000a, Jiggins *et al.*, 2001, Charlat *et al.*, 2005), Pseudoscorpiones (Zeh *et al.*, 2005, Zeh and Zeh, 2006), Hemiptera (Groeters, 1996), Hymenoptera (Werren *et al.*, 1986) and Coleoptera (Fialho and Stevens, 2000). The Coccinelidae seem particularly prone to male-killing agents as a high number of ladybird species has been shown to be infected with up to three different male-killing endosymbionts (Majerus and Hurst, 1997, Majerus and Majerus, 2000, Majerus, 2006, Majerus and Majerus, 2010a). The exact mechanism of male killing remains elusive, however,

evidence suggest that in *Drosophila* the bacteria targets the male sex-determining system (Veneti *et al*., 2005) and in the parasitoid wasp *Nasonia* the formation of centromeres is inhibited, causing cell development arrest (Ferree *et al*., 2008).

FEMINIZATION

Feminization acts by turning genetic males into phenotypic fertile females, constituting a high fitness advantage for the bacterium as a non-transmitting male is turned into a transmitting female. In contrast with male-killing, feminization does not affect the number of offspring produced by an infected female and results in equal amounts of offspring being produced by infected and uninfected females. Feminizing bacteria are present in the pillbug *Armadillidium vulgare*, where they cause hypertrophy of the androgenic gland resulting in the inhibited production of androgenic hormone, responsible for male development (Martin *et al*., 1999). This leads to the development of a phenotypic female with an underlying male genotype (Rousset *et al*., 1992, Juchault *et al*., 1993, Rigaud *et al*., 1999). Feminization has further been recorded from two insect species belonging to the Lepidoptera (Hiroki *et al*., 2002) and Hemiptera (Negri *et al*., 2006) and from a mite species (Weeks *et al*., 2001).

PARTHENOGENESIS INDUCTION

Parthenogenesis induction has been shown in species with a haplodiploid sex determination system such as mites (Weeks and Breeuwer, 2001), thrips (Arakaki *et al*., 2001) and Hymenoptera (Stouthamer *et a*l., 1990, Huigens and Stouthamer, 2003). In uninfected mothers, haploid males normally develop from unfertilized eggs while diploid females hatch from fertilized eggs. However, infected mothers produce all female broods in the absence of fertilization. This results from alterations in the embryonic development, which restores diploidy by interference in the cell cycle (Stouthamer and Kazmer, 1994, Pannebakker *et al*., 2004, Adachi-Hagimori *et al*., 2008) and fusion of nuclei (Gottlieb *et al*., 2002). As in feminization, a higher proportion of infected females is obtained, constituting a direct transmission advantage for the bacterium.

CYTOPLASMIC INCOMPATIBILITY (CI)

Cytoplasmic incompatibility is the phenomenon where sperm and egg cells are reproductively incompatible (Werren, 1997). Unidirectional CI is observed when infected males mate with uninfected females, causing high mortality rates in the offspring of these crosses. The reciprocal cross (uninfected males with infected females) and crosses between infected males and infected females yield normal numbers of viable offspring. Bidirectional CI occurs when males and females are infected with different, mutually incompatible strains of bacteria, resulting in higher offspring mortality as well. A modification/rescue model has been proposed in which bacteria cause a modification of male sperm that is subsequently counteracted by the bacterial production of a rescue factor in infected eggs. In the absence of such a rescue factor (i.e. in the absence of

infection in the egg cell) male sperm remains incompatible, resulting in an erroneous embryonic development (Werren, 1997). Cytological observations revealed that this is due to a delay in development of the male pronuclei in the first mitotic division causing the loss of paternal chromosomes (Tram and Sullivan, 2002, Serbus *et al.*, 2008). In diploids this causes reduced offspring viability through production of a haploid embryo and in haplodiploid species where bacterial modified sperm is only present in fertilized eggs developing as females, this causes female mortality or the production of haploid males, dependent on the level of paternal genome loss (Breeuwer and Werren, 1990, Breeuwer, 1997, Vavre *et al.*, 2000). Given that uninfected females experience a dramatic loss in offspring production if mated with an infected male, while infected females produce viable offspring when mated with both infected and uninfected males, CI strongly increases the proportion of infected females in the population. CI has been found in mites, isopods and most of the insect orders (detailed overview in Bourtzis and Miller, 2003) and is therefore considered to be the most widespread bacterial reproductive alteration.

OVERVIEW OF BACTERIA KNOWN TO ACT AS REPRODUCTIVE PARASITES

Currently five genera of bacteria have been found that can cause the above mentioned reproductive alterations in their arthropod host.

WOLBACHIA

(Class alpha-proteobacteria, Order Rickettsiales, Family Anaplasmataceae)

The alpha-proteobacterium *Wolbachia* was first observed in the ovaries of the mosquito *Culex pipiens* (Hertig and Wolbach, 1924). However, the first report of the effects of a *Wolbachia* infection and consequently the first report of a reproductive effect of endosymbiont bacteria took till 1971 when it was demonstrated that *Wolbachia* causes cytoplasmic incompatibility in mosquitoes (Yen and Barr, 1971). Since then it has been found that *Wolbachia* is the only endosymbiont species that can cause all of the above mentioned reproductive alterations in its host, i.e. male-killing, feminization, parthenogenesis induction and cytoplasmic incompatibility (Werren, 1997, Stouthamer *et al.*, 1999, Werren *et al.*, 2008, Engelstadter and Hurst, 2009; table 3). Moreover, it has been found to be extremely widespread as infections have been shown for all major insect orders, arachnids such as mites, spiders and scorpions, isopods and nematodes (Stouthamer *et al.*, 1999, Duron *et al.*, 2008a, Werren *et al.*, 2008, Engelstadter and Hurst, 2009) and meta-analysis of a large number of screening studies suggest that 66% of all arthropod species are infected with *Wolbachia* (Hilgenboecker *et al.*, 2008). Currently, 8 *Wolbachia* lineages are identified (commonly referred to as supergroup A-H), although their monophyletic status is debated (Baldo and Werren, 2007). Inside the host, *Wolbachia* infection is situated predominantly inside vacuoles in cells of the reproductive system although it has been observed to infect the somatic tissue as well (Dobson *et al.*, 1999). Besides obvious effects on host reproduction, negative effects on host fecundity have been found (Stouthamer and Luck, 1993, Brownlie *et al.*, 2009). Moreover, a shift of initial negative effects of *Wolbachia* infection towards a positive effect on host fecundity have been shown in natural populations of *Drosophila*

simulans over the course of twenty years (Weeks *et al*., 2007). Other effects were demonstrated such as increased protection against RNA viruses (Hedges *et al*., 2008) and a lowered transmission rate of dengue virus in mosquitoes (Bian *et al*., 2010). This led to the recent anthropogenic introduction and spread of CI inducing *Wolbachia* in natural populations to successfully suppress dengue transmission (Hoffmann *et al*., 2011, Walker *et al*., 2011).

RICKETTSIA

(Class alpha-proteobacteria, order Rickettsiales, family Rickettsiaceae)

Rickettsia are mostly known of the arthropod vectored diseases in vertebrates such as typhus and Rocky mountain fever (Raoult and Roux, 1997). However, insight into the range of effects of *Rickettsia* was altered by recognizing its effects on arthropod hosts (Perlman *et al*., 2006, Weinert *et al*., 2009). These are, among others, reduced fecundity in aphids (Chen *et al*., 2000, Sakurai *et al*., 2005), negative effects on their host dispersal behavior in the dwarf spider *Erigone atra* (Goodacre *et al*., 2009) and reproductive alterations in several insect species. *Rickettsia*, most notably in the same bacterial order as *Wolbachia*, has been shown to induce a strong male-killing phenotype in several ladybird (Majerus and Hurst, 1997, Majerus and Majerus, 2010a) and one buprestid species (Lawson *et al*., 2001) and parthenogenesis induction in Hymenoptera (Hagimori *et al*., 2006, Giorgini *et al*., 2010). To date, male-killing and parthenogenesis induction are the only reproductive alterations associated with *Rickettsia* as no cases are documented for feminization and cytoplasmic incompatibility (table 3). Similar to *Wolbachia*, *Rickettsia* is found within the cytoplasm of host cells, albeit not in vacuoles, of the reproductive and somatic tissue (Hurst *et al*., 1996a, Caspi-Fluger *et al*., 2011). One former *Rickettsia* species, *Orientia tsutsugamushi* (Tamura *et al*., 1995), the causative agent of scrub typhus, is suspected to suppress male development in a mite species as antibiotic treatment resulted in the production of a highly male biased sex ratio (Takahashi *et al*., 1997).

CARDINIUM

(Class Bacteroidetes, Order Bacteroidales, Family Bacteroidaceae)

Cardinium bacteria were only recently discovered to infect arthropods (Kurtti *et al*., 1996) and reproductive effects on their host were first documented for the false spider mite *Brevipalpus phoenicis* as *Cardinium* induced feminization was observed (Weeks *et al*., 2001). Furthermore, parthenogenesis induction and CI were demonstrated for several wasp and spider mite species (Hunter and Zchori-Fein, 2006 and references herein) demonstrating that *Cardinium* is able to cause multiple reproductive phenotypes and, together with *Wolbachia*, is the only endosymbiont known to induce feminization and cytoplasmic incompatibility (table 3). Screening studies revealed the presence of *Cardinium* bacteria in the Acari, Araenae, Opiliones, Hymenoptera and Hemiptera with specifically high frequencies in spiders and mites (Weeks *et al*., 2003, Zchori-Fein and Perlman, 2004, Duron *et al*., 2008b, Chang *et al*., 2010, Perlman *et al*., 2010). Recently, a new *Cardinium* group has been proposed to infect biting midges (Diptera, Nakamura *et al*., 2009). Other non-reproductive effects have been documented such as changes in host

oviposition behaviour (Zchori-Fein *et al*., 2001, Kenyon and Hunter, 2007) and increased fecundity (Weeks and Stouthamer, 2004). Similar to *Rickettsia*, *Cardinium* is found within the cytoplasm of host cells in the absence of vacuoles, and is shown to infect somatic and germline tissue (Kitajima *et al*., 2007).

SPIROPLASMA

(Class Mollicutes, Order Entomoplasmatales, Family Spiroplasmataceae)

Spiroplasma endosymbionts are most commonly associated with arthropods (most frequently with insects and ticks and to a lesser extent with Crustaceae) and plants where they can cause several pathogenic phenotypes (Ammar and Hogenhout, 2006, Regassa and Gasparich, 2006). Moreover, some of the arthropod associated *Spiroplasma* induce a sex ratio distortion in their host through the action of male-killing (table 3). Male-killing has first been observed in *Spiroplasma* infected *Drosophila* (Poulson and Sakaguchi, 1961, Williamson *et al*., 1999) and furthermore in several Lepidoptera (Jiggins *et al*., 2000a, Tabata *et al*., 2011) and ladybird species (Hurst *et al*., 1999, Majerus *et al*., 1999). *Spiroplasma* is found both inside somatic and reproductive tissue cells as well as extracellularly in the hemocoel (Bourtzis and Miller, 2003). The effects of many associations of *Spiroplasma* with arthropod hosts are currently unknown, but it seems that effects of *Spiroplasmas* may shift towards beneficial mutualisms such as enhanced host survival in the presence of parasitic wasps (Xie *et al*., 2010).

ARSENOPHONUS

(Class gamma-proteobacteria, Orde Enterobacteriales, Family Enterobacteriaceae)

Arsenophonus has first been identified as the son-killer trait in the parasitoid wasp *Nasonia vitripennis* (Skinner, 1985, Werren *et al*., 1986) where it causes the production of almost all female biased broods by the selective killing of males (Gherna *et al*., 1991). This is achieved through inhibiting the formation of maternal centromeres, which are necessary for male development (Ferree *et al*., 2008). Although screening studies showed a widespread distribution in diverse taxa of arthropods (overview in Wilkes *et al*., 2012), reproductive effects remain confined to the male-killing trait in *Nasonia* (table 3). Unlike the endosymbiont species described above, *Arsenophonus* is transmitted horizontally, while the other described endosymbiont show an almost exclusive vertical transmission through the cytoplasm of the egg cell. *Arsenophonus* is injected into the fly host by ovipositing females, after which it is ingested by the developing larvae and subsequently invades through the gut wall (Huger *et al*., 1985, Werren *et al*., 1986). This is reflected in the possibility of culturing *Arsenophonus* in cell-free media (Werren *et al*., 1986) and, more importantly, has been shown in natural populations to cause lateral transfer of bacteria to previously uninfected larvae (Duron *et al*., 2010).

	Male-killing	Feminization	Parthenogenesis induction	Cytoplasmic incompatibility (CI)
Wolbachia	x	x	x	x
Rickettsia	x		x	
Cardinium		x	x	x
Spiroplasma	x	x		
Arsenophonus	x			

Table 3. Summary of the reproductive effects associated with *Wolbachia*, *Rickettsia*, *Cardinium*, *Spiroplasma* and *Arsenophonus* infection (adopted from Engelstädter & Hurst, 2009).

This overview shows that the strategies employed by endosymbiont bacteria are diverse and have evolved at least several times independently, even within lineages (e.g. *Spiroplasma*). Moreover, bacterial taxa can differ in the frequency of occurrence of reproductive distorters from almost all currently documented members of the genus showing these capabilities (*Wolbachia*, *Cardinium*) to constituting only a small part (*Spiroplasma*). The same argumentation can be made for the diversity of reproductive phenotypes induced by a particular bacterial genus, with *Wolbachia* being the unsurpassed master manipulator (Werren *et al*., 2008). However, our current knowledge may be strongly biased towards the effects of *Wolbachia*, and the discovery of new endosymbiont species is only beginning to emerge. Nonetheless, due to the pronounced effects of these reproductive alterations and the high number of species infected with endosymbiont bacteria, endosymbionts are considered important actors in shaping their host reproductive biology and therefore evolutionary processes such as, among others, sex role reversal (Jiggins *et al*., 2000b), evolution of sex determination systems (Werren and Beukeboom, 1998, Caubet *et al*., 2000, Dedeine *et al*., 2001, Weeks *et al*., 2001, Cordaux *et al*., 2011) and speciation (Bordenstein *et al*., 2001, Jaenike *et al*., 2006).

GENETIC CONFLICT

Sex ratio biases in the presence of competition and cooperation (see above) can be regarded as adaptive as it maximizes individual fitness by reducing competition or enhancing the use of limited resources. However, the occurrence of a sex ratio bias induced by the presence of selfish genetic elements, i.e. endosymbiont bacteria, sex-chromosome meiotic drive and B-chromosomes, strongly opposes the Mendelian inheritance of the nuclear genes in the rest of the host genome. This difference in inheritance pattern generates intragenomic conflict between the selfish gene set and the host nuclear gene set (Cosmides and Tooby, 1981, Werren, 1987, Hurst, 1992, Hurst *et al.*, 1996b, Werren and Beukeboom, 1998). Therefore, in the presence of such selfish sex ratio distorters, strong selection is suspected to occur on the host genes to develop suppressing factors acting against the effects of the selfish element. In *Drosophila simulans* X-drive is caused by several X-linked loci targeting the Y-chromosome, which results in the production of an excess of females. However, in accordance with genetic conflict theory, this drive effect is countered by the action of suppressor genes on every major autosomal chromosome and on the Y-chromosome, subject to drive (Cazemajor *et al.*, 1997). B-chromosome suppression has been detected in the grasshopper *Eyprepocnemis plorans* and in the mealy bug *Pseudococcus obscurus* as genetic factors caused a reduction in transmission levels of the B-chromosome (Nur and Brett, 1985, Herrera *et al.*, 1996). Males of the ladybird species *Cheilomenes sexmaculata* are rescued from the effects of a male-killing endosymbiont by the presence of an autosomal suppressing gene (Majerus and Majerus, 2010b). Particularly well studied is the population dependent distribution of suppressor genes acting against the male-killing activity of a *Wolbachia* infection in the butterfly *Hypolimnas bolina* (Hornett *et al.*, 2006). Although individuals of all populations are infected with the same *Wolbachia* endosymbiont, populations located at the edge of its geographic distribution show a difference in the sex ratio produced by infected females. Interpopulation crosses revealed that this is due to presence of suppressor genes in certain populations, counteracting the endosymbiont effects and thus resulting in the production of equal sex ratios and masking the effect of the endosymbiont. In other populations these suppressor genes are absent and highly female biased sex ratios are produced.

These examples show that in line with genetic conflict theory host counteracting factors may evolve against the effects of sex ratio distorting selfish genetic elements. Moreover, the occurrence of such suppressor genes can render both the endosymbiont and their own effects quiescent.

ARANEAE: CURRENT STATUS

Sex determination in the Araneae order normally occurs through the presence of sex-chromosomes with males being the heterogametic sex and females the homogametic sex (see study species for a more detailed explanation). This results, after fertilization, in the production of an equal ratio of male and female offspring. Nevertheless, alternative reproductive phenotypes, seemingly contradicting the general pattern of a sex-chromosome based even

sex ratio, have been observed in several spider species. Currently, two types are identified, parthenogenesis induction and primary sex ratio distortion (overview in Goodacre *et al*., 2006, Martin and Goodacre, 2009). Parthenogenesis in the presence of males has been observed in the species *Dysdera hungarica* (Dysderidae, Deeleman-Reinhold, 1986, Rezac *et al*., 2007), in maleless populations of *Theotima minutissima* (Ochyroceratidae, Edwards *et al*., 2003) and is suggested to occur in *Coelotes* spp. (Amaurobiidae, Shimojana and Nishihira, 2000).
The absence of males, which could indicate parthenogenesis or a highly female biased sex ratio, has been found in *Anapistula caecula* (Symphytognathidae, Baert and Jocque, 1993) and in a *Hypognatha* spp. (Araneidae, Levi, 1996). A primary sex ratio distortion (sex ratio bias at the time of fertilization) is documented from the solitary dwarf spider *Pithyohyphantes phrygianus* (Linyphiidae, Gunnarsson and Andersson, 1992) and several social spider species belonging to different families (Eresidae, Theridiidae and Thomisidae, Aviles and Maddison, 1991, Rowell and Main, 1992, Aviles *et al*., 2000). This shows that the occurrence of alternative reproductive phenotypes has a rather wide distribution over several spider families,
and should not be considered as rare, isolated events. However, the underlying mechanism has received only little attention and remains largely unexplored. No cases are documented of sex-chromosome meiotic drive and although suspected supernumerary chromosomes are sometimes observed in karyotype studies of spiders, even in species with sex ratio distortion (Aviles and Maddison, 1991, Rowell and Main, 1992, Qingtao *et al*., 1996, Zhao *et al*., 2010, Stavale *et al*., 2011) their distribution and possible effects on reproduction remain unknown.
In contrast with sex-chromosome meiotic drive and B-chromosomes, infection with endosymbiont bacteria has received much more attention. A high number of screening studies revealed that all endosymbiont species, known to cause reproductive alterations in their host (i.e. *Wolbachia*, *Rickettsia*, *Cardinium*, *Spiroplasma* and *Arsenophonus*) are present in spiders (Rowley *et al*., 2004, Goodacre *et al*., 2006, Duron *et al*., 2008a, Duron *et al*., 2008b, Martin and Goodacre, 2009) and even that some endosymbionts (*Cardinium*) show a particular high frequency of infection (Duron *et al*., 2008b). Nevertheless, the effects of endosymbiont bacteria have been investigated in few cases. A non-reproductive effect has been shown in the dwarf spider *Erigone atra* as *Rickettsia* infected females show a lower propensity for long-distance dispersal (Goodacre *et al*., 2009). In another dwarf spider species *Pityohyphantes phrygianus* female post-copulatory position was found to influence offspring sex ratio (Gunnarsson and Andersson, 1996, Gunnarsson *et al*., 2004) resulting in female biased clutches. This relationship is dependent on female size and moreover seems to shift according to the *Wolbachia* infection status of both males and females, indicating an indirect effect of endosymbiont infection (Gunnarsson *et al*., 2009). Currently, no direct causal relationships between endosymbiont bacterial presence and reproductive effects in spiders are recorded.

STUDY SPECIES *OEDOTHORAX GIBBOSUS* (ERIGONINAE, LINYPHIIDAE, ARANEAE; BLACKWALL, 1841)

Currently 72 species are recognized in the dwarf spider genus *Oedothorax* (Platnick, 2012) of which five species i.e. *O. agrestis*, *O. apicatus*, *O. fuscus*, *O. retusus* and *O. gibbosus* (ARABEL, Baert, 1996) can be found in northwestern Europe, including Belgium. *O. apicatus* is a colonizing species that is often found in open, disturbed habitats such as agricultural fields while *O. agrestis* has a much more limited distribution, inhabiting shaded river banks. *O. retusus* is often found in a variety of wet habitats while *O. fuscus* is found predominantly in wet pastures. *Oedothorax gibbosus*, however, requires a constant wet environment and is therefore found in wet to very wet habitats with a stable groundwater level such as oligo- and mesotrophic alder carrs, wet meadows and peat bogs where it resides in leave litter and mosses, near the vicinity of open water (De Keer and Maelfait, 1989, Alderweireldt, 1992). It has a limited distribution in the northern part of Belgium where its prevalence has been extensively investigated and is classified as vulnerable according to the Red List for spiders of Flanders (Maelfait *et al.*, 1998). Females are typically larger than males in this species (Vanacker, 2005). In addition to this variation between the sexes, the species is quite unique as it shows a remarkable polymorphism within the male sex which is not encountered in the other *Oedothorax* species. Two male morphs are found differing in the presence of cephalic modifications. The *gibbosus* morph (figure 1a) is characterized by the presence of a conspicuous protuberance on the last third of the cephalothorax and a deep anterior groove lined with numerous, black setae. The *tuberosus* morph, however, lacks any such differentiations and has a flat carapace, resembling those of females (figure 1b). Previously it was assumed that both males belong to different species (*Oedothorax gibbosus*, Blackwall, 1841 and *Oedothorax tuberosus*, Blackwall, 1841) of which the females could not be discriminated phenotypically. However, it has been demonstrated that the two types of males resulted from a male dimorphism within a single species as both morphs hatched from the same egg sac (De Keer and Maelfait, 1988). Moreover, morphometric analysis revealed that the differences in male head structure represent two distinct morphs as no intermediates forms were found and all males could be clearly assigned to one of both phenotypes (Heinemann and Uhl, 2000). Laboratory crosses showed that the male dimorphism is determined by the presence of a diallelic gene which is not expressed in females (Maelfait *et al.*, 1990, Vanacker *et al.*, 2001). Expression of the dominant G allele, coding for a *gibbosus* morph (allele combination GG and Gg), results in the development of a hunch with hairy groove, while the recessive gene g codes for the development of a *tuberosus* morph (allele combination gg).

Marked differences exist between the two morphs in general life cycle characteristics. The juvenile phase (hatching from the egg sac till reaching adulthood) is longer for *gibbosus* males while the *tuberosus* morph has a longer adult phase (Vanacker, 2001). A higher adult lifespan for *tuberosus* was also detected under food stressed conditions and varying levels of humidity (Vanacker, 2003). Investigation of the reproductive output revealed that females copulated with a *tuberosus* male produce more viable egg sacs, of which more offspring hatch (Vanacker, 2005).

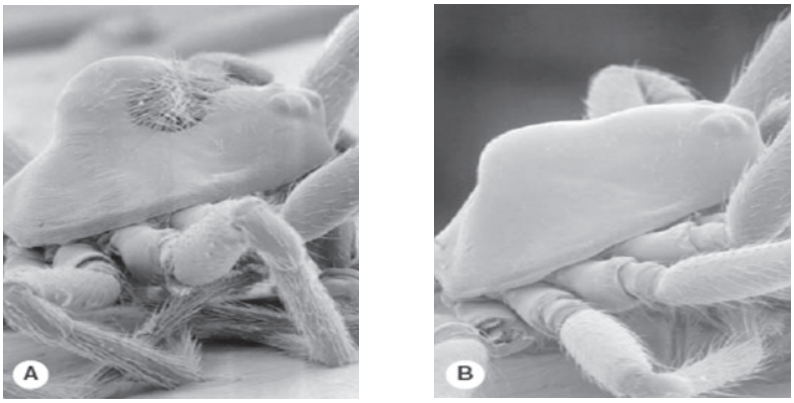


Figure 1. SEM photograph of the cephalothorax of a *gibbosus* (A) and *tuberosus* (B) male (Danny Vanacker, 2005).).

Moreover, it was inferred from laboratory matings that the difference in cephalic structures of the two morphs represents alternative mating strategies (as defined in Gross, 1996). For the first mating, females do not display any preference for either morph with the *gibbosus* and *tuberosus* morph showing equal acceptance rates (Vanacker *et al*., 2004). However, a strong preference exists towards the *gibbosus* male if the female is already inseminated as a high number of matings was observed of non-virgin females with a *gibbosus* male and almost no copulations occur with *tuberosus* males. This constitutes an advantage for the *gibbosus* male as sperm competition experiments demonstrated a clear last male sperm priority pattern (Vanacker, 2005). Most of the offspring of the first egg sac produced by a female are sired by the last male she mated with, after which last male sperm use steadily declines and more sperm of previous males is used. It is thought that the head structures of the *gibbosus* male are crucial for this preference by allowing the male to perform a gustatorial courtship. It was observed that before mating, females put their feeding parts in the groove of the male and exhibit feeding like behavior (Vanacker *et al*., 2003a). This strongly suggests the production of a secretion in the groove of the male which is offered as a nuptial gift, hence obtaining an additional copulation. This is supported by the observation of a high concentration of gland cells surrounding the hairy groove (Vanacker, 2005, Michalik and Uhl, 2011) and by interspecific interactions with a male from a related species, equally exhibiting feeding like behavior (Vanacker *et al*., 2003a, Vanacker *et al*., 2003b).

The persistence of such a male polymorphism in one population remains an evolutionary paradox as it is expected that strong sexual selection exists on these male traits affecting reproductive fitness (Oliveira *et al*., 2008). Especially in the case of *Oedothorax gibbosus*, where the dimorphism has a genetic basis, it is required that both morphs should have an equal fitness to be maintained in a stable manner (Shuster and Wade, 1991). This can be achieved if the male morphs are negatively frequency dependent selected, that is, if the relative fitness of a particular morph increases when its frequency is reduced in the population (Gadgil, 1972, Maynard Smith, 1982, Gross, 1996). However, empirical verification of the effect of frequency

dependent selection is currently lacking (Taborsky and Brockmann, 2011) as most studies focus on direct male-male competition (e.g. the competition between large males that fight to obtain copulations and smaller, sneaker males in beetle species (Emlen, 1997)) and do not incorporate the effect of female choice (Alonzo, 2008), which is likely to play an important role in *O. gibbosus*. Moreover, as inferred from theoretical approaches, the operational sex ratio (ratio of males and females, ready to mate in a population at any given time, Emlen and Oring, 1977, Kvarnemo and Ahnesjo, 1996) is expected to influence the dynamics of a male polymorphism due to its effect on the intensity of sexual selection. Particularly in *O. gibbosus*, it is expected that the operational sex ratio can alter the relative fitness of both morphs as the relative number of matings of both morphs is highly dependent on the number of inseminated females in the population (see above). As this number of inseminated females is expected to differ profoundly as a function of the operational sex ratio, this implies that the relative fitness of both morphs might alter accordingly (figure 2).

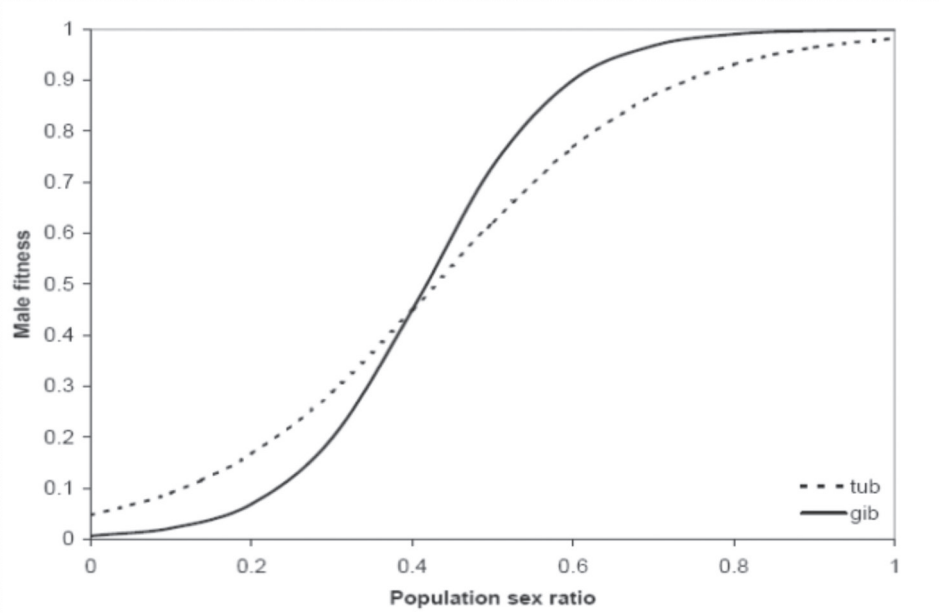


Figure 2. Relationship between population sex ratio and male fitness level. Striped line depicts the varying fitness of a *tuberosus* morph; full line represents a *gibbosus* morph.

If the population sex ratio is female biased a high proportion of virgin females is present and it can be suspected that, due to the shortage of males, most females will only mate once. These assumptions hold a fitness advantage for the *tuberosus* morph as virgin females show no preference for either morph and *tuberosus* males therefore will secure more matings than *gibbosus* males due to their faster juvenile development and longer adult lifespan. However, if the population sex ratio is male biased most females will mate multiple times and this constitutes

an advantage for the *gibbosus* morph as already inseminated females only accept *gibbosus* males for an additional mating, combined with the observed patterns of last male sperm priority. Preliminary theoretical efforts based on evolutionary game theory showed that if both males would be able to adjust their sex ratio, this system evolves towards an evolutionary stable strategy with *tuberosus* males producing a more female biased sex ratio compared to *gibbosus* males (Hendrickx and Mazalov, in prep). Previous breeding experiments performed by Vanacker (2004) similarly showed that *tuberosus* males tended to produce a more female biased sex ratio.

However, as sex determination in spiders occurs through the action of sex-chromosomes (Foelix, 1996) with the absence/presence of the sex-chromosomes determining the sex of an individual (Westergaard, 1958, Bull, 1983, Charlesworth, 1991) the production of an equal sex ratio is expected. In the case of *Oedothorax gibbosus*, males are characterized by the presence of one set of sex-chromosomes X1X2 (Král & Vanacker, unpublished results). Consequently, during spermatogenesis, two types of haploid sperm cells are produced in equal amounts: one type containing sex-chromosomes (X1X2-sperm) and a type characterized by the absence of sex-chromosomes (0-sperm). Females, on the other hand, develop when two sets of chromosomes are present, X1X2 X1X2. Because of this, one type of gamete is produced, containing one pair of sex-chromosomes, resulting, after fertilization, in the production of equal amounts of male and female offspring. Nevertheless, considerable variation in offspring sex ratio was observed in this species with the production of a significantly female biased sex ratio by some females (Vanacker *et al*., 2001, Vanacker, 2005), indicating the presence of a female biasing factor in this species.

Therefore, before investigating a potential link between sex ratio variation and the evolutionary dynamics of both male morphs, it is a prerequisite to first obtain a thorough insight into the mechanism behind the sex ratio variation in this species. This is the main objective of the current PhD study.

Spiders originated from two different sampling localities, the Walenbos and Damvallei of which previous work demonstrated the presence of *Oedothorax gibbosus* (De Knijf, 1993, Hendrickx, 1999).

The Walenbos is a large forest which is characterized by areas of water seepage resulting in the presence of isolated wet habitats such as alder carrs, providing a ideal habitat for *Oedothorax gibbosus*. This was confirmed in a sampling study as individuals of *O. gibbosus* were indeed found in every alder carr investigated, but not in the enclosing dry oak-birch forest areas (De Knijf, 1993). Within an alder carr, spiders can be found concentrated on tufts of moss isolated by open water. This indicates that *Oedothorax gibbosus* is highly habitat specific and only found under specific environmental conditions, which consequently results in a rather patchy distribution both within as well as among forest fragments. A comparable situation is found in the Damvallei, where a lowland marsh ecosystem arose by the formation of peat in a left meander of the river Schelde (Hendrickx, 1999). This equally results in the patchy presence of wet habitat where *Oedothorax gibbosus* can be found. In both localities spiders were sampled in one specific locality following a similar methodology. Spiders were collected through hand catches on a previously selected wet area of approximately 200 m^2, by, on average, four catchers for one hour. The same sampling location was used for consecutive sampling sessions. Previous research

suggested differences in *O. gibbosus* density as standardized hand catches revealed a higher number of individuals caught in the Walenbos compared to the Damvallei population (Patou, 2005). Moreover, it was observed that in the Damvallei population other species (i.e. *Oedothorax retusus*) were encountered in a higher frequency compared to the Walenbos sampling locality where almost exclusively *Oedothorax gibbosus* was caught (pers. obs.).

REFERENCES

- ADACHI-HAGIMORI, T., MIURA, K. & STOUTHAMER, R. 2008. A new cytogenetic mechanism for bacterial endosymbiont-induced parthenogenesis in Hymenoptera. *Proceedings of the Royal Society B-Biological Sciences*, 275, 2667-2673.
- ALDERWEIRELDT, M. 1992. Determinatieproblematiek van de zustersoorten van het genus *Oedothorax* (Araneae, Linyphiidae). *Newsbrief Belgische Arachnologische Vereniging*. 7, 4-8.
- ALONZO, S. H. 2008. Conflict between the sexes and alternative reproductive tactics within a sex. In: OLIVEIRA, R. F., TABORSKY, M. & BROCKMANN, H. J. (eds.) *Alternative reproductive tactics: an integrative approach*. Cambridge: Cambridge University Press.
- AMMAR, E. D. & HOGENHOUT, S. 2006. Mollicutes associated with arthropods and plants. *In:* BOURTZIS, K. & MILLER, T. A. (eds.) *Insect Symbiosis*. CRC Press.
- ARABEL ARADAT: Database of the Belgian Arachnofauna. online at: http://www.arabel.ugent.be/nl.php?page=data.
- ARAKAKI, N., MIYOSHI, T. & NODA, H. 2001. *Wolbachia*-mediated parthenogenesis in the predatory thrips *Fanklintothrips vespiformis* (Thysanoptera : Insecta). *Proceedings of the Royal Society of London Series B-Biological Sciences*, 268, 1011-1016.
- AVILES, L. & MADDISON, W. 1991. When Is the Sex-Ratio Biased in Social Spiders - Chromosome-Studies of Embryos and Male Meiosis in *Anelosimus* Species (Araneae, Theridiidae). *Journal of Arachnology*, 19, 126-135.
- AVILES, L., MCCORMACK, J., CUTTER, A. & BUKOWSKI, T. 2000. Precise, Highly Female-Biased Sex Ratios in a Social Spider. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 1445-1449.
- BAERT, L. 1996. Catalogus van de spinnen van België: Linyphiidae (Erigoninae) Deel 14.
- BAERT, L. & JOCQUE, R. 1993. *Anapistula caecula* n. sp., the smallest known female spider (Araneae, Symphytognathidae). *Journal of African Zoology*, 107, 187-189.
- BALDO, L. & WERREN, J. H. 2007. Revisiting *Wolbachia* supergroup typing based on *wsp*: spurious lineages and discordance with mlst. *Current Microbiology*, 55, 81-87.
- BANDI, C., DUNN, A. M., HURST, G. D. D. & RIGAUD, T. 2001. Inherited Microorganisms, Sex-Specific Virulence and Reproductive Parasitism. *Trends in Parasitology*, 17, 88-94.
- BELADJAL, L., VANDEKERCKHOVE, T. T. M., MUYSSEN, B., HEYRMAN, J., DE CAESEMAEKER, J. & MERTENS, J. 2002. B-Chromosomes and Male-Biased Sex Ratio With Paternal Inheritance in the Fairy Shrimp *Branchipus Schaefferi* (Crustacea, Anostraca). *Heredity*, 88, 356-360.
- BIAN, G. W., XU, Y., LU, P., XIE, Y. & XI, Z. Y. 2010. The Endosymbiotic Bacterium *Wolbachia* Induces Resistance to Dengue Virus in *Aedes aegypti*. *Plos Pathogens*, 6.
- BORDENSTEIN, S. R., O'HARA, F. P. & WERREN, J. H. 2001. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature*, 409, 707-710.
- BOURTZIS, K. & MILLER, T. A. 2003. *Insect Symbiosis*, CRC Press.
- BREEUWER, J. A. J. 1997. *Wolbachia* and cytoplasmic incompatibility in the spider mites *Tetranychus urticae* and *T-turkestani*. *Heredity*, 79, 41-47.
- BREEUWER, J. A. J. & WERREN, J. H. 1990. MICROORGANISMS ASSOCIATED WITH CHROMOSOME DESTRUCTION AND REPRODUCTIVE ISOLATION BETWEEN 2 INSECT SPECIES. *Nature*, 346, 558-560.

- BROWNLIE, J. C., CASS, B. N., RIEGLER, M., WITSENBURG, J. J., ITURBE-ORMAETXE, I., MCGRAW, E. A. & O'NEILL, S. L. 2009. Evidence for Metabolic Provisioning by a Common Invertebrate Endosymbiont, *Wolbachia pipientis*, during Periods of Nutritional Stress. *Plos Pathogens*, 5.
- BULL, J. J. 1983. *Evolution of sex determining mechanisms*, Menlo Park, Benjamin Cummings.
- BULMER, M. G. & TAYLOR, P. D. 1980. DISPERSAL AND THE SEX-RATIO. *Nature*, 284, 448-450.
- CAMACHO, J. P. M., SCHMID, M. & CABRERO, J. 2011. B Chromosomes and Sex in Animals. *Sexual Development*, 5, 155-166.
- CASPI-FLUGER, A., INBAR, M., MOZES-DAUBE, N., MOUTON, L., HUNTER, M. S. & ZCHORI-FEIN, E. 2011. *Rickettsia* 'In' and 'Out': Two Different Localization Patterns of a Bacterial Symbiont in the Same Insect Species. *Plos One*, 6.
- CAUBET, Y., HATCHER, M. J., MOCQUARD, J. P. & RIGAUD, T. 2000. Genetic conflict and changes in heterogametic mechanisms of sex determination. *Journal of Evolutionary Biology*, 13, 766-777.
- CAZEMAJOR, M., JOLY, D. & MONTCHAMP-MOREAU, C. 2000. Sex-Ratio Meiotic Drive in *Drosophila Simulans* Is Related to Equational Nondisjunction of the Y Chromosome. *Genetics*, 154, 229-236.
- CAZEMAJOR, M., LANDRE, C. & MONTCHAMPMOREAU, C. 1997. The sex-ratio trait in *Drosophila simulans*: Genetic analysis of distortion and suppression. *Genetics*, 147, 635-642.
- CHANG, J., MASTERS, A., AVERY, A. & WERREN, J. H. 2010. A divergent *Cardinium* found in daddy long-legs (Arachnida: Opiliones). *Journal of Invertebrate Pathology*, 105, 220-227.
- CHARLAT, S., HORNETT, E. A., DYSON, E. A., HO, P. P. Y., LOC, N. T., SCHILTHUIZEN, M., DAVIES, N., RODERICK, G. K. & HURST, G. D. D. 2005. Prevalence and penetrance variation of male-killing *Wolbachia* across Indo-Pacific populations of the butterfly *Hypolimnas bolina*. *Molecular Ecology*, 14, 3525-3530.
- CHARLAT, S., HURST, G. D. D. & MERCOT, H. 2003. Evolutionary Consequences of *Wolbachia* Infections. *Trends in Genetics*, 19, 217-223.
- CHARLESWORTH, B. 1991. THE EVOLUTION OF SEX-CHROMOSOMES. *Science*, 251, 1030-1033.
- CHARNOV, E. L. 1982. *The theory of sex allocation*, Princeton University Press.
- CHEN, D. Q., MONTLLOR, C. B. & PURCELL, A. H. 2000. Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A-kondoi*. *Entomologia Experimentalis Et Applicata*, 95, 315-323.
- CORDAUX, R., BOUCHON, D. & GREVE, P. 2011. The impact of endosymbionts on the evolution of host sex-determination mechanisms. *Trends in Genetics*, 27, 332-341.
- COSMIDES, L. M. & TOOBY, J. 1981. CYTOPLASMIC INHERITANCE AND INTRAGENOMIC CONFLICT. *Journal of Theoretical Biology*, 89, 83-129.
- DAWKINS, R. 1976. *The Selfish Gene*, Oxford University Press.
- DE BARY, A. 1879. *The Phenomenon of Symbiosis*, Strassbourg.
- DE BRITO PORTELA-CASTRO, A. L., JULIO, H. F. J. & BELINI NISHIYAMA, P. 2001. New occurrence of microchromosomes B in *Moenkhausia sanctaefilomenae* (Pisces, Characidae) from the Parana River of Brazil: analysis of the synaptonemal complex. *Genetica*, 110, 277-283.
- DE KEER, R. & MAELFAIT, J.-P. 1988. *Oedothorax gibbosus* (Blackwall) and Oedothorax tuberosus (Blackwall): one species. *Newsletter of the British Arachnological Society*, 53.
- DE KEER, R. & MAELFAIT, J.-P. 1989. Ecological backgrounds for the distribution patterns of *Oedothorax fuscus* (Blackwall) and *Oedothorax gibbosus* (Blackwall) (Erigoninae, Araneae). *Proceedings of the Symposium "Invertebrates of Belgium"*, 189-192.

- DE KNIJF, G. 1993. *Aspekten van de ekologie van de spinnenfauna (Araneae) van het Walenbos te Tielt-Winge (Vlaams-Brabant)*. Master thesis, Ghent University.
- DEDEINE, F., VAVRE, F., FLEURY, F., LOPPIN, B., HOCHBERG, M. E. & BOULETREAU, M. 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 6247-6252.
- DEELEMAN-REINHOLD, C. D. 1986. *Dysdera hungarica* Kulczynski, a case of parthenogenesis? *Actas X International Arachnological Congress, Spain*, 1, 25-31.
- DOBSON, S. L., BOURTZIS, K., BRAIG, H. R., JONES, B. F., ZHOU, W. G., ROUSSET, F. & O'NEILL, S. L. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochemistry and Molecular Biology*, 29, 153-160.
- DOOLITTLE, W. F. & SAPIENZA, C. 1980. SELFISH GENES, THE PHENOTYPE PARADIGM AND GENOME EVOLUTION. *Nature*, 284, 601-603.
- DOUGLAS, A. E. 1998. Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology*, 43, 17-37.
- DURON, O., BOUCHON, D., BOUTIN, S., BELLAMY, L., ZHOU, L. Q., ENGELSTADTER, J. & HURST, G. D. 2008a. The Diversity of Reproductive Parasites Among Arthropods: *Wolbachia* Do Not Walk Alone. *BMC Biology*, 6:27.
- DURON, O., HURST, G. D. D., HORNETT, E. A., JOSLING, J. A. & ENGELSTÄDTER, J. 2008b. High incidence of the maternally inherited bacterium *Cardinium* in spiders. *Molecular Ecology*, 1-11.
- DURON, O., WILKES, T. E. & HURST, G. D. D. 2010. Interspecific transmission of a male-killing bacterium on an ecological timescale. *Ecology Letters*, 13, 1139-1148.
- EDWARDS, R. L., EDWARDS, E. H. & EDWARDS, A. D. 2003. Observations of *Theotima Minutissimus* (Araneae, Ochyroceratidae), a Parthenogenetic Spider. *Journal of Arachnology*, 31, 274-277.
- ELNAGDY, S., MAJERUS, M. E. N. & HANDLEY, L. J. L. 2011. The value of an egg: resource reallocation in ladybirds (Coleoptera: Coccinellidae) infected with male-killing bacteria. *Journal of Evolutionary Biology*, 24, 2164-2172.
- EMLEN, D. J. 1997. Alternative reproductive tactics and male-dimorphism in the horned beetle *Ontophagus acuminatus* (Coleoptera: Scarabaeidae). B*ehavioral Ecology and Sociobiology*, 41, 335-341.
- EMLEN, S. T. & ORING, L. W. 1977. ECOLOGY, SEXUAL SELECTION, AND EVOLUTION OF MATING SYSTEMS. *Science*, 197, 215-223.
- ENGELSTADTER, J. & HURST, G. D. D. 2009. The Ecology and Evolution of Microbes That Manipulate Host Reproduction. *Annual Review of Ecology Evolution and Systematics*, 40, 127-149.
- FERREE, P. M., AVERY, A., AZPURUA, J., WILKES, T. & WERREN, J. H. 2008. A bacterium targets maternally inherited centrosomes to kill males in *Nasonia*. *Current Biology*, 18, 1409-1414.
- FIALHO, R. F. & STEVENS, L. 2000. Male-Killing *Wolbachia* in a Flour Beetle. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 1469-1473.
- FISHER, R. A. 1930. *The genetical theory of natural selection*., Oxford University Press.
- FOELIX, R. F. 1996. *Biology of Spiders*, Oxford University Press.
- FREGDA, K., GROPP, A., WINKING, H. & FRANK, F. 1976. A hypothesis explaining the exceptional sex ratio in the wood lemming (*Myopus schisticolor*). *Hereditas*, 85, 101-104.

- FREGDA, K., GROPP, A., WINKING, H. & FRANK, F. 1977. Fertile XX- and XY-type females in the wood lemming *Myopus schisticolor*. *Nature*, 261, 225-227.
- GADGIL, M. 1972. Male dimorphism as a consequence of sexual selection. *Am. Nat.*, 106, 574-580.
- GERSHENSON, S. 1928. A new sex-ratio abnormality in *Drosophila obscura*. *Genetics*, 13, 488-507.
- GHERNA, R. L., WERREN, J. H., WEISBURG, W., COTE, R., WOESE, C. R., MANDELCO, L. & BRENNER, D. J. 1991. *ARSENOPHONUS-NASONIAE* GEN-NOV, SP-NOV, THE CAUSATIVE AGENT OF THE SON-KILLER TRAIT IN THE PARASITIC WASP *NASONIA-VITRIPENNIS*. International *Journal of Systematic Bacteriology*, 41, 563-565.
- GIORGINI, M., BERNARDO, U., MONTI, M. M., NAPPO, A. G. & GEBIOLA, M. 2010. *Rickettsia* Symbionts Cause Parthenogenetic Reproduction in the Parasitoid Wasp *Pnigalio soemius* (Hymenoptera: Eulophidae). *Applied and Environmental Microbiology*, 76, 2589-2599.
- GOODACRE, S. L., MARTIN, O. Y., BONTE, D., HUTCHINGS, L., WOOLLEY, C., IBRAHIM, K., THOMAS, C. F. G. & HEWITT, G. M. 2009. Microbial modification of host long-distance dispersal capacity. *BMC Biology*, 7:32.
- GOODACRE, S. L., MARTIN, O. Y., THOMAS, C. F. G. & HEWITT, G. M. 2006. *Wolbachia* and Other Endosymbiont Infections in Spiders. *Molecular Ecology*, 15, 517-527.
- GOTTLIEB, Y., ZCHORI-FEIN, E., WERREN, J. H. & KARR, T. L. 2002. Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera : pteromalidae). *Journal of Invertebrate Pathology*, 81, 166-174.
- GROETERS, F. R. 1996. Maternally Inherited Sex Ratio Distortion as a Result of a Male-Killing Agent in *Spilostethus hospes* (Hemiptera: Lygaeidae). *Heredity*, 77, 201-208.
- GROSS, M. R. 1996. Alternative Reproductive Strategies and Tactics: Diversity Within Sexes (Vol 11, Pg 92, 1996). *Trends in Ecology & Evolution*, 11, 263.
- GUNNARSSON, B. & ANDERSSON, A. 1992. CHROMOSOME VARIATION IN EMBRYOS OF A SOLITARY SPIDER, *PITYOHYPHANTES-PHRYGIANUS*, WITH SKEWED SEX-RATIO. *Hereditas*, 117, 85-91.
- GUNNARSSON, B. & ANDERSSON, A. 1996. Sex Ratio Variation in Sheet-Web Spiders: Options for Female Control? *Proceedings of the Royal Society of London Series B-Biological Sciences*, 263, 1177-1182.
- GUNNARSSON, B., GOODACRE, S. L. & HEWITT, G. M. 2009. Sex Ratio, Mating Behaviour and *Wolbachia* Infections in a Sheetweb Spider. *Biological Journal of the Linnean Society*, 98, 181-186.
- GUNNARSSON, B., UHL, G. & WALLIN, K. 2004. Variable Female Mating Positions and Offspring Sex Ratio in the Spider *Pityohyphantes phrygianus* (Araneae : Linyphiidae). *Journal of Insect Behavior*, 17, 129-144.
- HAGIMORI, T., ABE, Y., DATE, S. & MIURA, K. 2006. The first finding of a *Rickettsia* bacterium associated with parthenogenesis induction among insects. *Current Microbiology*, 52, 97-101.
- HAMILTON, W. D. 1967. EXTRAORDINARY SEX RATIOS. *Science*, 156, 477-&.
- HEDGES, L. M., BROWNLIE, J. C., O'NEILL, S. L. & JOHNSON, K. N. 2008. *Wolbachia* and Virus Protection in Insects. *Science*, 322, 702.
- HEINEMANN, S. & UHL, G. 2000. Male Dimorphism in *Oedothorax gibbosus* (Araneae, Linyphiidae): a Morphometric Analysis. *Journal of Arachnology*, 28, 23-28.

- HENDRICKX, F. 1999. De spinnenfauna van het laagveengebied de "Damvallei" te Destelbergen (Oost-Vlaanderen). *Nwsbr. Belg. Arachnol. Ver.*, 14.
- HERRERA, J. A., LOPEZLEON, M. D., CABRERO, J., SHAW, M. W. & CAMACHO, J. P. M. 1996. Evidence for B chromosome drive suppression in the grasshopper *Eyprepocnemis plorans*. *Heredity*, 76, 633-639.
- HERTIG, M. & WOLBACH, S. B. 1924. Studies on *rickettsia*-like microorganisms in insects. *J. Med. Res.*, 44, 329-374.
- HICKEY, W. A. & CRAIG, G. B. 1966. Genetic distortion of sex ratio in *Aedes* aegypti. *Genetics*, 53, 1177-1196.
- HILGENBOECKER, K., HAMMERSTEIN, P., SCHLATTMANN, P., TELSCHOW, A. & WERREN, J. H. 2008. How Many Species Are Infected With *Wolbachia*? - A Statistical Analysis of Current Data. *Fems Microbiology Letters*, 281, 215-220.
- HIROKI, M., KATO, Y., KAMITO, T. & MIURA, K. 2002. Feminization of genetic males by a symbiotic bacterium in a butterfly, *Eurema hecabe* (Lepidoptera : Pieridae). *Naturwissenschaften*, 89, 167-170.
- HOFFMANN, A. A., MONTGOMERY, B. L., POPOVICI, J., ITURBE-ORMAETXE, I., JOHNSON, P. H., MUZZI, F., GREENFIELD, M., DURKAN, M., LEONG, Y. S., DONG, Y., COOK, H., AXFORD, J., CALLAHAN, A. G., KENNY, N., OMODEI, C., MCGRAW, E. A., RYAN, P. A., RITCHIE, S. A., TURELLI, M. & O'NEILL, S. L. 2011. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature*, 476, 454-U107.
- HORNETT, E. A., CHARLAT, S., DUPLOUY, A. M. R., DAVIES, N., RODERICK, G. K., WEDELL, N. & HURST, G. D. D. 2006. Evolution of Male-Killer Suppression in a Natural Population. *Plos Biology*, *4*, 1643-1648.
- HUGER, A. M., SKINNER, S. W. & WERREN, J. H. 1985. BACTERIAL-INFECTIONS ASSOCIATED WITH THE SON-KILLER TRAIT IN THE PARASITOID WASP *NASONIA*-(= *MORMONIELLA*)-*VITRIPENNIS* (HYMENOPTERA, PTEROMALIDAE). *Journal of Invertebrate Pathology*, 46, 272-280.
- HUIGENS, M. E. & STOUTHAMER, R. 2003. Parthenogenesis associated with *Wolbachia*. *In:* BOURTZIS, K. & MILLER, T. A. (eds.) *Insect Symbiosis*. CRC Press.
- HUNTER, M. S., NUR, U. & WERREN, J. H. 1993. ORIGIN OF MALES BY GENOME LOSS IN AN AUTOPARASITOID WASP. *Heredity*, 70, 162-171.
- HUNTER, M. S. & ZCHORI-FEIN, E. 2006. Inherited Bacteroidetes symbionts in arthropods. *In:* BOURTZIS, K. & MILLER, T. A. (eds.) *Insect Symbiosis*. CRC Press.
- HURST, G. D. D. & JIGGINS, F. M. 2000. Male-Killing Bacteria in Insects: Mechanisms, Incidence, and Implications. *Emerging Infectious Diseases*, 6, 329-336.
- HURST, G. D. D. & MAJERUS, M. E. N. 1993. WHY DO MATERNALLY INHERITED MICROORGANISMS KILL MALES. *Heredity*, 71, 81-95.
- HURST, G. D. D., VON DER SCHULENBURG, J. H. G., MAJERUS, T. M. O., BERTRAND, D., ZAKHAROV, I. A., BAUNGAARD, J., VOLKL, W., STOUTHAMER, R. & MAJERUS, M. E. N. 1999. Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Molecular Biology*, 8, 133-139.
- HURST, G. D. D., WALKER, L. E. & MAJERUS, M. E. N. 1996a. Bacterial infections of hemocytes associated with the maternally inherited male-killing trait in British populations of the two spot ladybird, *Adalia bipunctata*. *Journal of Invertebrate Pathology*, 68, 286-292.

- HURST, G. D. D. & WERREN, J. H. 2001. The Role of Selfish Genetic Elements in Eukaryotic Evolution. *Nature Reviews Genetics*, 2, 597-606.
- HURST, L. D. 1992. INTRAGENOMIC CONFLICT AS AN EVOLUTIONARY FORCE. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 248, 135-140.
- HURST, L. D., ATLAN, A. & BENGTSSON, B. O. 1996b. Genetic conflicts. *Quarterly Review of Biology*, 71, 317-364.
- IMAI, H. T. 1974. B-CHROMOSOMES IN MYRMICINE ANT, *LEPTOTHORAX-SPINOSIOR*. *Chromosoma*, 45, 431-444.
- JAENIKE, J. 2001. Sex Chromosome Meiotic Drive. *Annual Review of Ecology and Systematics*, 32, 25-49.
- JAENIKE, J., DYER, K. A., CORNISH, C. & MINHAS, M. S. 2006. Asymmetrical reinforcement and *Wolbachia* infection in *Drosophila*. *Plos Biology*, 4, 1852-1862.
- JIGGINS, F. M., HURST, G. D. D., JIGGINS, C. D., VON DER SCHULENBURG, J. H. G. & MAJERUS, M. E. N. 2000a. The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. *Parasitology*, 120, 439-446.
- JIGGINS, F. M., HURST, G. D. D. & MAJERUS, M. E. N. 2000b. Sex-Ratio-Distorting *Wolbachia* Causes Sex-Role Reversal in Its Butterfly Host. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 69-73.
- JIGGINS, F. M., HURST, G. D. D., SCHULENBURG, J. & MAJERUS, M. E. N. 2001. Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. *Heredity*, 86, 161-166.
- JORDAL, B. H., BEAVER, R. A., NORMARK, B. B. & FARRELL, B. D. 2002. Extraordinary sex ratios and the evolution of male neoteny in sib-mating *Ozopemon* beetles. Biological *Journal of the Linnean Society*, 75, 353-360.
- JUCHAULT, P., RIGAUD, T. & MOCQUARD, J. P. 1993. EVOLUTION OF SEX DETERMINATION AND SEX-RATIO VARIABILITY IN WILD POPULATIONS OF *ARMADILLIDIUM-VULGARE* (LATR) (CRUSTACEA, ISOPODA) - A CASE-STUDY IN CONFLICT-RESOLUTION. *Acta Oecologica-International Journal of Ecology*, 14, 547-562.
- KENYON, S. G. & HUNTER, M. S. 2007. Manipulation of oviposition choice of the parasitoid wasp, *Encarsia pergandiella*, by the endosymbiotic bacterium *Cardinium*. *Journal of Evolutionary Biology*, 20, 707-716.
- KIRKENDALL, L. R. 1993. Ecology and evolution of biased sex ratios in bark and ambrosia beetles. *In:* WRENSCH, D. L. & EBBERT, M. A. (eds.) *Evolution and diversity of sex ratio in insects and mites*. New York: Chapman and Hall.
- KITAJIMA, E. W., GROOT, T. V. M., NOVELLI, V. M., FREITAS-ASTUA, J., ALBERTI, G. & DE MORAES, G. J. 2007. In situ observation of the *Cardinium* symbionts of *Brevipalpus* (Acari : Tenuipalpidae) by electron microscopy. *Experimental and Applied Acarology*, 42, 263-271.
- KOMDEUR, J. 1996. Facultative sex ratio bias in the offspring of Seychelles warblers. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 263, 661-666.
- KOMDEUR, J., DAAN, S., TINBERGEN, J. & MATEMAN, C. 1997. Extreme adaptive modification in sex ratio of the Seychelles warbler's eggs. *Nature*, 385, 522-525.
- KUDO, T. 2009. Termite-Microbe Symbiotic System and Its Efficient Degradation of Lignocellulose. *Bioscience Biotechnology and Biochemistry*, 73, 2561-2567.

- KURTTI, T. J., MUNDERLOH, U. G., ANDREADIS, T. G., MAGNARELLI, L. A. & MATHER, T. N. 1996. Tick cell culture isolation of an intracellular prokaryote from the tick *Ixodes scapularis*. *Journal of Invertebrate Pathology*, 67, 318-321.
- KVARNEMO, C. & AHNESJO, I. 1996. The Dynamics of Operational Sex Ratios and Competition for Mates. *Trends in Ecology & Evolution*, 11, 404-408.
- LAWSON, E. T., MOUSSEAU, T. A., KLAPER, R., HUNTER, M. D. & WERREN, J. H. 2001. *Rickettsia* associated with male-killing in a buprestid beetle. *Heredity*, 86, 497-505.
- LEVI, H. W. 1996. The American orb-weavers *Hypognatha*, *Encyosaccus*, *Xylethrus*, *Gasteracantha* and *Enacrosoma* (Araneae, Araneidae). *Bulletin of the Museum of comparative Zoology Cambridge*, 155, 89-157.
- LYTTLE, T. W. 1991. Segregation Distorters. *Annual Review of Genetics*, 25, 511-557.
- MACKE, E., MAGALHAES, S., BACH, F. & OLIVIERI, I. 2011. Experimental Evolution of Reduced Sex Ratio Adjustment Under Local Mate Competition. *Science*, 334, 1127-1129.
- MAELFAIT, J.-P., BAERT, L., JANSSEN, M. & ALDERWEIRELDT, M. 1998. A Red list for the spiders of Flanders. *Bulletin of the Royal Institute of Natural Sciences*, Entomology, 131-142.
- MAELFAIT, J.-P., DE KEER, R. & DE MEESTER, L. 1990. Genetical background of the polymorphism of *Oedothorax gibbosus* (Blackwall) (Linyphiidae, Araneae). *Revue Arachnologique*, 9, 29-34.
- MAJERUS, M. E. N. 2003. *Sex Wars* Princeton University Press.
- MAJERUS, M. E. N. 2006. The impact of male-killing bacteria on the evolution of aphidophagous coccinellids. *European Journal of Entomology*, 103, 1-7.
- MAJERUS, M. E. N. & HURST, G. D. D. 1997. Ladybirds as a model system for the study of male-killing symbionts. *Entomophaga*, 42, 13-20.
- MAJERUS, M. E. N. & MAJERUS, T. M. O. 2000. Female-biased sex ratio due to male-killing in the Japanese ladybird *Coccinula sinensis*. *Ecological Entomology*, 25, 234-238.
- MAJERUS, T. M. O. & MAJERUS, M. E. N. 2010a. Discovery and identification of a male-killing agent in the Japanese ladybird *Propylea japonica* (Coleoptera: Coccinellidae). *BMC Evolutionary Biology*, 10:37.
- MAJERUS, T. M. O. & MAJERUS, M. E. N. 2010b. Intergenomic Arms Races: Detection of a Nuclear Rescue Gene of Male-Killing in a Ladybird. *Plos Pathogens*, 6.
- MAJERUS, T. M. O., VON DER SCHULENBURG, J. H. G., MAJERUS, M. E. N. & HURST, G. D. D. 1999. Molecular Identification of a Male-Killing Agent in the Ladybird *Harmonia axyridis* (Pallas) (Coleoptera : Coccinellidae). *Insect Molecular Biology*, 8, 551-555.
- MARTIN, G., SOROKINE, O., MONIATTE, M., BULET, P., HETRU, C. & VAN DORSSELAER, A. 1999. The structure of a glycosylated protein hormone responsible for sex determination in the isopod, *Armadillidium vulgare*. *European Journal of Biochemistry*, 262, 727-736.
- MARTIN, M. M. 1991. THE EVOLUTION OF CELLULOSE DIGESTION IN INSECTS. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 333, 281-288.
- MARTIN, O. Y. & GOODACRE, S. L. 2009. Widespread Infections by the Bacterial Endosymbiont *Cardinium* in Arachnids. *Journal of Arachnology*, 37, 106-108.
- MAYNARD SMITH, J. 1982. *Evolution and the theory of games*., Cambridge, Cambridge University Press.
- MCKEE, B. D. 1998. Pairing sites and the role of chromosome pairing in meiosis and spermatogenesis in male *Drosophila*. *Meiosis and Gametogenesis*, 37, 77-115.

- MICHALIK, P. & UHL, G. 2011. Cephalic Modifications in Dimorphic Dwarf Spiders of the Genus *Oedothorax* (Erigoninae, Linyphiidae, Araneae) and Their Evolutionary Implications. *Journal of Morphology*, 272, 814-832.
- MONTCHAMP-MOREAU, C. & JOLY, D. 1997. Abnormal spermiogenesis is associated with the X-linked sex-ratio trait in *Drosophila simulans*. *Heredity*, 79, 24-30.
- MORGAN-RICHARDS, M. 2000. Robertsonian translocations and B chromosomes in the Wellington tree weta, *Hemideina crassidens* (Orthoptera : Anostostomatidae). *Hereditas*, 132, 49-54.
- NAKAMURA, Y., KAWAI, S., YUKUHIRO, F., ITO, S., GOTOH, T., KISIMOTO, R., YANASE, T., MATSUMOTO, Y., KAGEYAMA, D. & NODA, H. 2009. Prevalence of *Cardinium* Bacteria in Planthoppers and Spider Mites and Taxonomic Revision of "Candidatus *Cardinium hertigii*" Based on Detection of a New *Cardinium* Group from Biting Midges. *Applied and Environmental Microbiology*, 75, 6757-6763.
- NEGRI, I., PELLECCHIA, M., MAZZOGLIO, P. J., PATETTA, A. & ALMA, A. 2006. Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/XO sex-determination system. *Proceedings of the Royal Society B-Biological Sciences*, 273, 2409-2416.
- NEO, D. M., MOREIRA, O. & CAMACHO, J. P. M. 2000. Altitudinal variation for B chromosome frequency in the characid fish *Astyanax scabripinnis*. *Heredity*, 85, 136-141.
- NUR, U. & BRETT, B. L. H. 1985. GENOTYPES SUPPRESSING MEIOTIC DRIVE OF A B-CHROMOSOME IN THE MEALYBUG, *PSEUDOCOCCUS-OBSCURUS*. *Genetics*, 110, 73-92.
- NUR, U., WERREN, J. H., EICKBUSH, D. G., BURKE, W. D. & EICKBUSH, T. H. 1988. A SELFISH B-CHROMOSOME THAT ENHANCES ITS TRANSMISSION BY ELIMINATING THE PATERNAL GENOME. *Science*, 240, 512-514.
- OHKUMA, M. 2008. Symbioses of flagellates and prokaryotes in the gut of lower termites. *Trends in Microbiology*, 16, 345-352.
- OLIVEIRA, R. F., TABORSKY, M. & BROCKMANN, H. J. 2008. *Alternative reproductive tactics: an integrative approach*., Cambridge, Cambridge University Press.
- ORGEL, L. E. & CRICK, F. H. C. 1980. SELFISH DNA - THE ULTIMATE PARASITE. *Nature*, 284, 604-607.
- ÖSTERGREN, G. 1945. Parasitic nature of extra fragment chromosomes. *Bot. Not.*, 2, 157-163.
- PANNEBAKKER, B. A., PIJNACKER, L. P., ZWAAN, B. J. & BEUKEBOOM, L. W. 2004. Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera : Figitidae). *Genome*, 47, 299-303.
- PATOU, E. 2005. *Interdemic variation in sex ratio en in the relative proportion of the two morphs in the male dimorphic dwarf spider Oedothorax gibbosus (Blackwall, 1841) (Erigoninae, Linyphiidae, Araneae).* Master thesis, Ghent University.
- PERLMAN, S. J., HUNTER, M. S. & ZCHORI-FEIN, E. 2006. The emerging diversity of *Rickettsia*. *Proceedings of the Royal Society B-Biological Sciences*, 273, 2097-2106.
- PERLMAN, S. J., MAGNUS, S. A. & COPLEY, C. R. 2010. Pervasive associations between *Cybaeus* spiders and the bacterial symbiont *Cardinium*. *Journal of Invertebrate Pathology*, 103, 150-155.
- PLATNICK, N. I. 2012. The world spider catalog, version 12.5. American Museum of Natural History. online at http://research.amnh.org/iz/spiders/catalog. DOI: 10.5531/db.iz.0001.
- POULSON, D. F. & SAKAGUCHI, B. 1961. NATURE OF SEX-RATIO AGENT IN *DROSOPHILA*. *Science*, 133, 1489-&.

- QINGTAO, Z., JIANZHANG, Y. & FENGXIANG, L. 1996. Study on the B chromosomes of spider I. B chromosomes of *Clubiona* japonicola (Clubionidae, Araneidae) in Wuhan. *Acta Arachnologica Sinica*, 5, 132-136.
- RAOULT, D. & ROUX, V. 1997. Rickettsioses as paradigms of new or emerging infectious diseases. *Clinical Microbiology Reviews*, 10, 694-&.
- REGASSA, L. B. & GASPARICH, G. E. 2006. *Spiroplasmas*: evolutionary relationships and biodiversity. *Frontiers in Bioscience*, 11, 2983-3002.
- REZAC, M., KRAL, J. & PEKAR, S. 2007. The spider genus *Dysdera* (Araneae, Dysderidae) in central Europe: Revision and natural history. *Journal of Arachnology*, 35, 432-462.
- RIGAUD, T., BOUCHON, D., SOUTY-GROSSET, C. & RAIMOND, P. 1999. Mitochondrial Dna Polymorphism, Sex Ratio Distorters and Population Genetics in the Isopod *Armadillidium vulgare*. *Genetics*, 152, 1669-1677.
- ROUSSET, F., BOUCHON, D., PINTUREAU, B., JUCHAULT, P. & SOLIGNAC, M. 1992. *WOLBACHIA* ENDOSYMBIONTS RESPONSIBLE FOR VARIOUS ALTERATIONS OF SEXUALITY IN ARTHROPODS. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 250, 91-98.
- ROWELL, D. M. & MAIN, B. Y. 1992. Sex-Ratio in the Social Spider *Diaea-Socialis* (Araneae, Thomisidae). *Journal of Arachnology*, 20, 200-206.
- ROWLEY, S. M., RAVEN, R. J. & MCGRAW, E. A. 2004. *Wolbachia pipientis* in Australian Spiders. *Current Microbiology*, 49, 208-214.
- SAKURAI, M., KOGA, R., TSUCHIDA, T., MENG, X. Y. & FUKATSU, T. 2005. *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: Novel cellular tropism, effect on host fitness, and interaction with the essential symbiont *Buchnera*. *Applied and Environmental Microbiology*, 71, 4069-4075.
- SERBUS, L. R., CASPER-LINDLEY, C., LANDMANN, F. & SULLIVAN, W. 2008. The Genetics and Cell Biology of *Wolbachia*-Host Interactions. *Annual Review of Genetics*, 42, 683-707.
- SHIMOJANA, M. & NISHIHIRA, M. 2000. A new cave-dwelling eyeless spider of the genus *Coelotes* (Araneae: Amaurobidae) from Okinawa island, the Ryukyu Islands, Japan, with notes on possible parthenogenesis. *Acta Arachnologica Tokyo*, 49, 29-40.
- SHUSTER, S. M. & WADE, M. J. 1991. Equal mating succes among male reproductive strategies in a marine isopod. *Nature*, 350, 608-610.
- SKINNER, S. W. 1985. Son-Killer - a 3rd Extrachromosomal Factor Affecting the Sex-Ratio in the Parasitoid Wasp, *Nasonia* (=*Mormoniella*) *vitripennis*. *Genetics*, 109, 745-759.
- STAVALE, L. M., SCHNEIDER, M. C., BRESCOVIT, A. D. & CELLA, D. M. 2011. Chromosomal characteristics and karyotype evolution of Oxyopidae spiders (Araneae, Entelegynae). *Genetics and Molecular Research*, 10, 752-763.
- STOUTHAMER, R., BREEUWER, J. A. J. & HURST, G. D. D. 1999. *Wolbachia pipientis*: Microbial Manipulator of Arthropod Reproduction. *Annual Review of Microbiology*, 53, 71-102.
- STOUTHAMER, R., HURST, G. D. D. & BREEUWER, J. A. J. 2002. Sex ratio distorters and their detection. *I.C.W. Hardy, Sex ratios: concepts and research methods* Cambridge University Press.
- STOUTHAMER, R. & KAZMER, D. J. 1994. CYTOGENETICS OF MICROBE-ASSOCIATED PARTHENOGENESIS AND ITS CONSEQUENCES FOR GENE FLOW IN *TRICHOGRAMMA* WASPS. *Heredity*, 73, 317-327.

- STOUTHAMER, R. & LUCK, R. F. 1993. INFLUENCE OF MICROBE-ASSOCIATED PARTHENOGENESIS ON THE FECUNDITY OF *TRICHOGRAMMA-DEION* AND *T-PRETIOSUM*. *Entomologia Experimentalis Et Applicata*, 67, 183-192.
- STOUTHAMER, R., LUCK, R. F. & HAMILTON, W. D. 1990. ANTIBIOTICS CAUSE PARTHENOGENETIC *TRICHOGRAMMA* (*HYMENOPTERA*, TRICHOGRAMMATIDAE) TO REVERT TO SEX. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 2424-2427.
- STOUTHAMER, R., VAN TILBORG, M., DE JONG, J. H., NUNNEY, L. & LUCK, R. F. 2001. Selfish element maintains sex in natural populations of a parasitoid wasp. *Proceedings of the Royal Society of London Series B-Biological Sciences,* 268, 617-622.
- TABATA, J., HATTORI, Y., SAKAMOTO, H., YUKUHIRO, F., FUJII, T., KUGIMIYA, S., MOCHIZUKI, A., ISHIKAWA, Y. & KAGEYAMA, D. 2011. Male Killing and Incomplete Inheritance of a Novel *Spiroplasma* in the Moth *Ostrinia zaguliaevi*. *Microbial Ecology*, 61, 254-263.
- TABORSKY, M. & BROCKMANN, H. J. 2011. Alternative reproductive tactics and life history phenotypes. *In:* KAPPELER, P. (ed.) *Animal Behaviour: evolution and mechanisms*. Heidelberg: Springer.
- TAKAHASHI, M., URAKAMI, H., YOSHIDA, Y., FURUYA, Y., MISUMI, H., HORI, E., KAWAMURA, A. & TANAKA, H. 1997. Occurrence of high ratio of males after introduction of minocycline in a colony of *Leptotrombidium fletcheri* infected with *Orientia tsutsugamushi*. *European Journal of Epidemiology*, 13, 79-86.
- TAMURA, A., OHASHI, N., URAKAMI, H. & MIYAMURA, S. 1995. CLASSIFICATION OF *RICKETTSIA-TSUTSUGAMUSHI* IN A NEW GENUS, ORIENTIA GEN-NOV, AS *ORIENTIA-TSUTSUGAMUSHI* COMB-NOV. *International Journal of Systematic Bacteriology*, 45, 589-591.
- TAYLOR, D. R. 1999. Genetics of sex ratio variation among natural populations of a dioecious plant. *Evolution*, 53, 55-62.
- TAYLOR, P. D. 1981. INTRA-SEX AND INTER-SEX SIBLING INTERACTIONS AS SEX-RATIO DETERMINANTS. *Nature*, 291, 64-66.
- TAYLOR, P. D. & BULMER, M. G. 1980. LOCAL MATE COMPETITION AND THE SEX-RATIO. *Journal of Theoretical Biology*, 86, 409-419.
- TRAM, U. & SULLIVAN, W. 2002. Rate of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science*, 296, 1124-1126.
- TRIVERS, R. L. & WILLARD, D. E. 1973. NATURAL-SELECTION OF PARENTAL ABILITY TO VARY SEX-RATIO OF OFFSPRING. *Science*, 179, 90-92.
- VANACKER, D. 2001. The male dimorphism in the dwarf spider *Oedothorax gibbosus* (Blackwall, 1841)(Erigoninae, Linyphiidae, Araneae): Results of laboratory rearing experiments. *Belgian Journal of Zoology*, 131, 39-44.
- VANACKER, D. 2003. Survival differences of the two male morphs in the dwarf spider *Oedothorax gibbosus* (Blackwall, 1841)(Erigoninae, Linyphiidae, Araneae). *Netherlands Journal of Zoology*, 52, 255-262.
- VANACKER, D. 2005. *Morphology, function and evolution of the male cephalic structures of the dwarf spider genus Oedothorax*. PhD thesis, Ghent University.
- VANACKER, D., HENDRICKX, F., MAES, L., VERRAES, P. & MAELFAIT, J. P. 2004. Can multiple mating compensate for slower development and shorter adult life in a male dimorphic dwarf spider? *Biological Journal of the Linnean Society*, 82, 269-273.

- VANACKER, D., MAELFAIT, J.-P. & BAERT, L. 2001. The male dimorphism in the dwarf spider *Oedothorax gibbosus* (Blackwall, 1841) (Erigoninae, Linyphiidae, Araneae): Results of laboratory rearing experiments. *Belg. J. Zool.*, 131, 39-44.
- VANACKER, D., MAES, L., PARDO, S., HENDRICKX, F. & MAELFAIT, J. P. 2003a. Is the hairy groove in the *gibbosus* male morph of *Oedothorax gibbosus* (Blackwall 1841) a nuptial feeding device? *Journal of Arachnology*, 31, 309-315.
- VANACKER, D., VANDEN BORRE, J., JONCKHEERE, A., MAES, L., PARDO, S., HENDRICKX, F. & MAELFAIT, J. P. 2003b. Dwarf spiders (Erigoninae, Linyphiidae, Araneae): good candidates for evolutionary research. *Belgian Journal of Zoology*, 133, 143-149.
- VAVRE, F., FLEURY, F., VARALDI, J., FOUILLET, P. & BOULETREAU, M. 2000. Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: Epidemiologic and evolutionary consequences. *Evolution*, 54, 191-200.
- VENETI, Z., BENTLEY, J. K., KOANA, T., BRAIG, H. R. & HURST, G. D. D. 2005. A functional dosage compensation complex required for male killing in *Drosophila*. *Science*, 307, 1461-1463.
- VICENTE, V. E., MOREIRA, O. & CAMACHO, J. P. M. 1996. Sex-ratio distortion associated with the presence of a B chromosome in *Astyanax scabripinnis* (Teleostei, Characidae). *Cytogenetics and Cell Genetics*, 74, 70-75.
- WALKER, T., JOHNSON, P. H., MOREIRA, L. A., ITURBE-ORMAETXE, I., FRENTIU, F. D., MCMENIMAN, C. J., LEONG, Y. S., DONG, Y., AXFORD, J., KRIESNER, P., LLOYD, A. L., RITCHIE, S. A., O'NEILL, S. L. & HOFFMANN, A. A. 2011. The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature*, 476, 450-U101.
- WEEKS, A. R. & BREEUWER, J. A. J. 2001. *Wolbachia*-induced parthenogenesis in a genus of phytophagous mites. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 268, 2245-2251.
- WEEKS, A. R., MAREC, F. & BREEUWER, J. A. J. 2001. A mite species that consists entirely of haploid females. *Science*, 292, 2479-2482.
- WEEKS, A. R. & STOUTHAMER, R. 2004. Increased fecundity associated with infection by a Cytophaga-like intracellular bacterium in the predatory mite, *Metaseiulus* occidentalis. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 271, S193-S195.
- WEEKS, A. R., TURELLI, M., HARCOMBE, W. R., REYNOLDS, K. T. & HOFFMANN, A. A. 2007. From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *Plos Biology*, 5, 997-1005.
- WEEKS, A. R., VELTEN, R. & STOUTHAMER, R. 2003. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, 1857-1865.
- WEINERT, L. A., WERREN, J. H., AEBI, A., STONE, G. N. & JIGGINS, F. M. 2009. Evolution and diversity of *Rickettsia* bacteria. *BMC Biology*, 7:6.
- WERREN, J. H. 1987. THE COEVOLUTION OF AUTOSOMAL AND CYTOPLASMIC SEX-RATIO FACTORS. *Journal of Theoretical Biology*, 124, 317-334.
- WERREN, J. H. 1997. Biology of *Wolbachia*. *Annual Review of Entomology*, 42, 587-609.
- WERREN, J. H. 2011. Selfish genetic elements, genetic conflict, and evolutionary innovation. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 10863-10870.
- WERREN, J. H., BALDO, L. & CLARK, M. E. 2008. *Wolbachia*: Master Manipulators of Invertebrate Biology. *Nature Reviews Microbiology*, 6, 741-751.

- WERREN, J. H. & BEUKEBOOM, L. W. 1998. Sex Determination, Sex Ratios, and Genetic Conflict. *Annual Review of Ecology and Systematics*, 29, 233-261.
- WERREN, J. H., NUR, U. & WU, C. I. 1988. SELFISH GENETIC ELEMENTS. *Trends in Ecology & Evolution*, 3, 297-302.
- WERREN, J. H. & O'NEILL, S. L. 1997. The evolution of heritable symbionts. *In:* O'NEILL,
- S. L. (ed.) I*nfluential Passengers, Inherited Microorganisms and Arthropod Reproduction*. Oxford University Press.
- WERREN, J. H., SKINNER, S. W. & CHARNOV, E. L. 1981. PATERNAL INHERITANCE OF A DAUGHTERLESS SEX-RATIO FACTOR. *Nature*, 293, 467-468.
- WERREN, J. H., SKINNER, S. W. & HUGER, A. M. 1986. MALE-KILLING BACTERIA IN A PARASITIC WASP. *Science*, 231, 990-992.
- WERREN, J. H. & STOUTHAMER, R. 2003. PSR (paternal sex ratio) chromosomes: the ultimate selfish genetic elements. *Genetica*, 117, 85-101.
- WEST, S. A. 2009. *Sex Allocation*, Princeton University Press.
- WESTERGAARD, M. 1958. THE MECHANISM OF SEX DETERMINATION IN DIOECIOUS FLOWERING PLANTS. *Advances in Genetics Incorporating Molecular Genetic Medicine*, 9, 217-281.
- WILKES, T., DURON, O., DARBY, C., HYPSA, V., NOVAKOVA, E. & HURST, G. D. D. 2012. The Genus *Arsenophonus*. *In:* ZCHORI-FEIN, E. & BOURTZIS, K. (eds.) *Manipulative Tenants*. CRC Press.
- WILKINSON, G. S. & SANCHEZ, M. I. 2001. Sperm development, age and sex chromosome meiotic drive in the stalk-eyed fly, *Cyrtodiopsis whitei*. *Heredity*, 87, 17-24.
- WILLIAMSON, D. L. & POULSON, D. F. 1979. Sex ratio organisms (*Spiroplasmas*) of *Drosophila* In: WHITCOMB, R. F. & TULLY, J. G. (eds.) *The Mycocplasmas*. Academic Press, New York.
- WILLIAMSON, D. L., SAKAGUCHI, B., HACKETT, K. J., WHITCOMB, R. F., TULLY, J. G., CARLE, P., BOVE, J. M., ADAMS, J. R., KONAI, M. & HENEGAR, R. B. 1999. *Spiroplasma poulsonii* sp. nov., a new species associated with male-lethality in *Drosophila willistoni*, a neotropical species of fruit fly. *International Journal of Systematic Bacteriology*, 49, 611-618.
- XIE, J. L., VILCHEZ, I. & MATEOS, M. 2010. *Spiroplasma* Bacteria Enhance Survival of *Drosophila hydei* Attacked by the Parasitic Wasp *Leptopilina heterotoma*. *Plos One*, 5.
- YEN, J. H. & BARR, A. R. 1971. NEW HYPOTHESIS OF CAUSE OF CYTOPLASMIC INCOMPATIBILITY IN *CULEX-PIPIENS* L. *Nature*, 232, 657-&.
- YOSHIDA, K., TERAI, Y., MIZOIRI, S., AIBARA, M., NISHIHARA, H., WATANABE, M., KUROIWA, A., HIRAI, H., HIRAI, Y., MATSUDA, Y. & OKADA, N. 2011. B Chromosomes Have a Functional Effect on Female Sex Determination in Lake Victoria Cichlid Fishes. *Plos Genetics*, 7.
- ZCHORI-FEIN, E., GOTTLIEB, Y., KELLY, S. E., BROWN, J. K., WILSON, J. M., KARR, T. L. & HUNTER, M. S. 2001. A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 12555-12560.
- ZCHORI-FEIN, E. & PERLMAN, S. J. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. *Molecular Ecology*, 13, 2009-2016.
- ZEH, D. W., ZEH, J. A. & BONILLA, M. M. 2005. *Wolbachia*, sex ratio bias and apparent male killing in the harlequin beetle riding pseudoscorpion. *Heredity*, 95, 41-49.
- ZEH, J. A. & ZEH, D. W. 2006. Male-killing *Wolbachia* in a live-bearing arthropod: Brood abortion as a constraint on the spread of a selfish microbe. *Journal of Invertebrate Pathology*, 92, 33-38.

• ZHAO, Y., AYOUB, N. A. & HAYASHI, C. Y. 2010. Chromosome Mapping of Dragline Silk Genes in the Genomes of Widow Spiders (Araneae, Theridiidae). *Plos One*, 5.

OBJECTIVES AND THESIS OUTLINE

This thesis aims at providing a mechanistic insight in the evolution of distorted sex ratios in the Araneae order. Although several species are known to exhibit a significantly distorted sex ratio, the underlying mechanisms remain at present elusive. As study organism, we selected the male dimorphic species *Oedothorax gibbosus*, known to exhibit considerable sex ratio variation. Moreover, given that changes in the population sex ratio are expected to strongly influence traits related to sexual selection, investigating the mechanisms of sex ratio variation in this species allows future studies to address the relationship between sex ratio and male dimorphic evolutionary dynamics. Here, we aim to:

(i) quantify sex ratio variation in this species and explore the inheritance pattern of this sex ratio trait.

(ii) investigate the role of endosymbiont bacteria as a causative agent for the observed sex ratio variation.

(iii) explore if a genetic conflict occurs between the sex ratio distorting factor and the host's nuclear genes.

(iv) investigate if the phenomenon of sex ratio distortion is confined to this rather unique male dimorphic species, or if it is also present in a closely related species that lacks this male dimorphism.

Within **chapter 1** we investigate the inheritance pattern of the sex ratio bias as this provides information on the possible causative agent of the sex ratio distortion. We fit an animal model to a large pedigree containing several generations of lab rearing and information of thousands of offspring. The role of endosymbiont bacteria as a causative agent is further explored by performing specific PCR's to characterize the endosymbiont community in *Oedothorax gibbosus* and apply an antibiotics treatment to test if the sex ratio trait has a bacterial basis.

In chapter 1 we found that individual spiders can be infected with up to three different endosymbiont species, known to cause reproductive alterations in their host i.e. *Wolbachia*, *Rickettsia* and *Cardinium*. Although a significant relationship was found between Wolbachia infection and the occurrence of a female distorted sex ratio bias, several distorted sex ratios were observed in the absence of *Wolbachia* as well. This strongly suggests that an additional factor causes a female bias in this species. In **chapter 2** we explore this further by concentrating on those individuals that are not infected with *Wolbachia*. Several molecular tools and antibiotics treatments are used in order to pinpoint the exact identity of possible endosymbionts present.

As obligate intracellular endosymbiont bacteria are almost exclusively maternally inherited, conflict with the biparental inheritance of the host genes predicts the evolution of host suppressor genes that counteract the effects of the endosymbiont. In **chapter 3** we investigate the presence of such nuclear suppressor genes by performing inter- and intrapopulation crosses of two isolated populations, and test for intrapopulation variation among males in their ability to suppress the bacterial phenotype.

This is also touched upon in **chapter 4**, where we investigated the possibility of sex-chromosome meiotic drive by flow cytometric sexing of spider sperm.

In **chapter 5**, we move beyond the male dimorphic study species and test if the same factor that causes sex ratio variation in *Oedothorax gibbosus* is also present in the related species, *Oedothorax retusus*, which lacks the occurrence of multiple male phenotypes.

“I’m tough,
I’m ambitious,
and I know exactly
what I want.
If that makes me
a bitch, okay.”

Madonna ‘Wolbachia’ Ciccone

CHAPTER 1

SPIDERS DO NOT ESCAPE REPRODUCTIVE MANIPULATIONS BY *WOLBACHIA*

BRAM VANTHOURNOUT [1]

JANNE SWAEGERS [1]

FREDERIK HENDRICKX [1,2]

Adapted from Vanthournout, B., Swaegers, J. & Hendrickx, F. Spiders do not escape reproductive manipulations by *Wolbachia*. BMC Evolutionary Biology 2011 11:15.

[1] **Terrestrial Ecology Unit, Department of Biology, Ghent University, Ledeganckstraat 35, 9000 Ghent, Belgium**

[2] **Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium**

ABSTRACT

Maternally inherited bacteria that reside obligatorily or facultatively in arthropods can increase their prevalence in the population by altering their hosts' reproduction. Such reproductive manipulations have been reported from the major arthropod groups such as insects (in particular hymenopterans, butterflies, dipterans and beetles), crustaceans (isopods) and mites. Despite the observation that endosymbiont bacteria are frequently encountered in spiders and that the sex ratio of particular spider species is strongly female biased, a direct relationship between bacterial infection and sex ratio variation has not yet been demonstrated for this arthropod order. Females of the dwarf spider *Oedothorax gibbosus* exhibit considerable variation in the sex ratio of their clutches and were infected with at least three different endosymbiont bacteria capable of altering host reproduction i.e. *Wolbachia*, *Rickettsia* and *Cardinium*. Breeding experiments show that sex ratio variation in this species is primarily maternally inherited and that removal of the bacteria by antibiotics restores an unbiased sex ratio. Moreover, clutches of females infected with *Wolbachia* were significantly female biased while uninfected females showed an even sex ratio. As female biased clutches were of significantly smaller size compared to non-distorted clutches, killing of male embryos appears to be the most likely manipulative effect. This represents to our knowledge the first direct evidence that endosymbiont bacteria, and in particular *Wolbachia*, might induce sex ratio variation in spiders. These findings are pivotal to further understand the diversity of reproductive phenotypes observed in this arthropod order.

1. INTRODUCTION

Maternally inherited endosymbiont bacteria of arthropods received considerable attention owing to their ability to shape their hosts' reproductive biology and consequently their ecology and evolution (Werren and Beukeboom, 1998, Charlat *et al*., 2003, Werren *et al*., 2008, Engelstädter and Hurst, 2009). The strategies adopted by these microorganisms to increase their fitness are surprisingly diverse and involve the induction of parthenogenesis, killing of male offspring, feminization of genetic males and cytoplasmic incompatibility, wherein, in its simplest form, the development of an uninfected egg is inhibited if inseminated by sperm of an infected male (Stouthamer *et al*., 1999, Werren *et al*., 2008, Engelstädter and Hurst, 2009).

Recently, several molecular screening studies demonstrated that the degree of arthropod infection is considerably higher than previously thought (Jeyaprakash and Hoy, 2000, Duron *et al*., 2008a) and that even up to 66% of arthropod species are thought to be infected (Hilgenboecker *et al*., 2008). Despite the discovery of their widespread occurrence within arthropods, knowledge about the extent to which they may alter their hosts' reproduction is lagging behind. Even for some large taxonomic groups, such as the order of spiders (Araneae), conclusive evidence is at present lacking. Nevertheless, some spider species exhibit reproductive phenotypes similar to those expected under endosymbiont infection such as parthenogenesis and primary sex ratio distortion in social as well as solitary species (e.g. Avilès, 1986, Deeleman-Reinhold, 1986, Rowell and Main, 1992, Camacho, 1994, Avilès *et al*., 2000, Edwards *et al*., 2003, Gunnarsson *et al*., 2004) (and see Martin and Goodacre, 2009 for an extensive overview). In particular for solitary species, primary sex ratio distortion is commonly expected to result from reproductive manipulation by endosymbionts as Fisher's sex-allocation theory generally predicts that an equal sex ratio is the only evolutionary stable outcome from the host's perspective. Moreover, the prevalence and diversity of endosymbiont bacteria in spiders is among the highest within the arthropods and of up to five different endosymbionts capable of manipulating their hosts' reproductive biology have been found in several spider families: *Wolbachia*, *Rickettsia*, *Cardinium*, *Arsenophonus* and *Spiroplasma* (Oh *et al*., 2000, Rowley *et al*., 2004, Goodacre *et al*., 2006, Duron *et al*., 2008a, Duron *et al*., 2008b, Martin and Goodacre, 2009).

Causal relationships between endosymbiont infection and sex ratio distortion in spiders are up till now only suggested by a difference in their prevalence between males and females (Duron *et al*., 2008a) or by an indirect relationship (Gunnarsson *et al*., 2009). Yet, as many other factors beside endosymbionts might cause sex ratio distortion (Werren and Beukeboom, 1998), multiple lines of evidence such as maternal inheritance of sex ratio variation, use of different antibiotics that target an array of different bacterial families and a direct relationship between endosymbiont presence and sex ratio effect are necessary to disentangle the impact of each endosymbiont on the produced sex ratio (Stouthamer *et al*., 1999, Weeks *et al*., 2002).

In this study, we report on sex ratio variation in the solitary spider *Oedothorax gibbosus* (Araneae: Linyphiidae: Erigoninae). This small dwarf spider has a palearctic distribution and occurs exclusively in damp habitats such as marshes and wet forests, where they reside in grass tussocks and patches of moss situated close to the water. Besides the observation that the species

exhibits a clear male dimorphism with alternative mating strategies (Vanacker *et al.*, 2004), previous research showed primary sex ratio distortion with an excess of females (Vanacker, 2004). Here, we explore the potential role of endosymbionts in inducing this sex ratio variation by (i) unraveling the inheritance pattern of the sex ratio trait, (ii) relating the presence of several endosymbiont bacteria with sex ratio variation and (iii) investigating whether an equal sex ratio can be restored by antibiotic treatments.

2. MATERIAL AND METHODS

2.1 MATERNAL INHERITANCE OF SEX RATIO VARIATION

To estimate the among female variance in clutch sex ratio and decompose it into a maternally inherited and residual component, a total of 192 females were captured in a wet forest ("Walenbos", Belgium) and mated with males from the same population. Offspring were bred for five consecutive generations in the lab, which resulted in a total of 3884 offspring originating from 414 different females. Spiders were reared individually in plastic vials of 5 cm diameter and 2, 5 cm height. Plaster was added to the bottom and moistened to keep humidity levels at 100 %. A piece of moss was provided to allow the construction of a functional web. The vials were placed in a climate chamber with a constant temperature of 20°C and light-dark regime of 16L-8D. Juveniles were fed with an overabundance of springtails (*Sinella curviseta*) and after the third moult an excess of fruit flies (*Drosophila* sp.) was provided. Vials were checked several times a week for food and humidity level. After reaching adulthood one male was placed in the vial of the female. The male was removed after at least 24h. Mated females were allowed to lay cocoons in the vial they were reared in. Sex was determined upon reaching adulthood by visual inspection using a stereomicroscope and tertiary sex ratio, defined as number of male offspring divided by the total number of adult offspring, was assessed.

Presence of a maternally inherited sex ratio variation and an estimate of its variance (σ_m^2) over multiple generations was obtained by means of an animal model (Lynch and Walsh, 1998) in which maternal effects were updated using Gibbs sampling. It is an extension of a model developed by (Damgaard, 2007) to estimate additive genetic effects for continuous traits in pedigrees.
More specifically, let Y_i be the sex of offspring *i* and let *d,i* refer to the mother of the *i*'th offspring, then Y_i was modelled following a Bernoulli distribution with mean $\pi_{d,i}$, i.e. the sex ratio of the dam *d*, where

$$\ln\left(\frac{\pi_{d,i}}{1-\pi_{d,i}}\right) = \mu + m_{d,i} + e_{d,i}$$

with μ the average logit sex ratio in the population, $m_{d,i}$ the maternally inherited sex ratio effect and $e_{d,i}$ the sex ratio effect that is not captured in $m_{d,i}$. Possible causes of $e_{d,i}$ to deviate from zero includes additional variation in sex ratio among dams that is not attributed to sampling error. As sex ratio data are obtained over multiple generations in the pedigree, $m_{d,i}$ can be updated separately from $e_{d,i}$ by means of a recursive equation that sets the maternally inherited sex ratio effect of a particular mother equal to that of her daughters, i.o.w. $m_i = m_{d,i}$. Hence, $m_{d,i}$ of a particular dam is updated from both the sex ratio she produces as well as the sex ratio that her (grand)mother(s) and (grand)daughters produce. Following this procedure, the posterior distribution of the variance in $m_{d,i}$ and $e_{d,i}$, i.e. σ_m^2 and σ_e^2 respectively, was obtained and used to calculate a point estimate based on the mean of the distribution and a 95% credibility interval (CI). The model was fitted using a Bayesian approach as implemented in the program WinBugs

v.1.4. A gamma (0.1, 0.1) was chosen as a prior distribution for σ_m and σ_e. Two independent MCMC chains, each with different starting values, were run simultaneously for 12.000 generations. The first 2.000 generations were discarded as burn-in period.

We also fitted a more complex model that included a genetic component that contributes to the sex ratio variation. However, visual inspection of the independent MCMC's indicated that no convergence and, hence, no reliable estimates of the variance components could be obtained. To explore which manipulation is involved, we correlated clutch size and clutch sex ratio. Ideally, killing of male offspring by endosymbiont bacteria reduces the clutch size to half the clutch size produced by non-manipulated females. Feminization and parthenogenesis on the other hand are expected to have no effect on clutch size. We related clutch size and clutch sex ratio by means of a Pearson correlation, wherein the estimate of the clutch sex ratio was weighed by the clutch size in order to down weight the inaccurate sex ratio estimates of small clutches.

2.2 ENDOSYMBIONT DETECTION AND PREVALENCE

Infection status and screening of endosymbionts was performed by means of PCR (Polymerase Chain Reaction) with endosymbiont specific primers. DNA was extracted from whole spiders with the Nucleospin Tissue kit (©Machery Nagel) following the manufacturers recommended protocol.

Spiders were screened for four different endosymbionts that are known to alter the hosts reproduction in arthropods and that are already detected in spiders by means of the following specific primers: (i) WSP81F and WSP691R (Braig *et al*., 1998) to amplify a part of the cell surface protein coding gene of *Wolbachia* (*wsp*) and 16Swolb99F and 16Swolb994R (O'Neill *et al*., 1992) to selectively amplify the 16S ribosomal RNA gene of *Wolbachia*; (ii) CLO-f1 and CLO-r1 (Gotoh *et al*., 2007) to selectively amplify ~468bp part of the 16S rRNA gene of *Cardinium*; (iii) RICS741F and RICT1197R, which amplify a part of the *citrate* gene of *Rickettsia* (Davis *et al*., 1998, Majerus *et al*., 2000) and (iv) SP-ITS-J04 and SP-ITS-N55 (Majerus *et al*., 1999) to selectively amplify the spacer region between 16S and 23S rRNA genes of *Spiroplasma ixodetis*. To investigate whether *Wolbachia* detection in our study species was not confounded by the presence of *Wolbachia* in prey items, one sample of three fruitflies and one of 20 springtails were screened. None of these samples showed any traces of *Wolbachia*. PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing (54 °C, 30 s), extension (72 °C, 90 s) and a final extension at 72 °C during 5 min. Electrophoresis was performed on a 1,5% agarose gel. The primers 3F and 9R (Giribet *et al*., 1996), which amplify part of the 18S rDNA, were used as a positive control. Gels were stained in a solution of GELRED and bands were visualized by UV-fluorescence.

PCR products were sequenced using BigDye Terminator Sequencing mix and run on an ABI 3710 automated sequencer for a random sample of five to ten independent individuals to validate primer specificity and to test for the presence of different strains. Sequences were aligned with the ClustalW algorithm implemented in MEGA4 (Tamura *et al*., 2007) with sequences from other

spiders available in GenBank (mainly reported in the studies Goodacre *et al.*, 2006, Duron *et al.*, 2008a, Duron *et al.*, 2008b). The closest relatives of the obtained endosymbiont sequences were identified by BLAST searches. Phylogenetic position and similarity of the endosymbionts with those of other spider species were analyzed based on a Bayesian Inference using MrBayes (Huelsenbeck and Ronquist, 2001) with posterior probability support values calculated for the nodes. We assumed a general time reversible model of DNA substitution allowing a proportion of invariant sites and gamma distributed variation in substitution rate among sites (GTR+I+G) for all four DNA fragments. Four simultaneous chains (two cold, two heated) were run for two million generations and trees were sampled every 1000 generations. To check convergence and stability of the parameter estimates and to determine the burn-in value, we used Tracer 1.3 (Rambaut and Drummond, 2007) to inspect the log files.

Prevalence of the bacteria was tested in two populations in Belgium, i.e. "Walenbos", being the same locality where spiders of the breeding experiment (i) originated from ($n = 53$ and $n = 11$ females and males respectively) and a second population "Damvallei" situated approximately 100km westward ($n = 39$ and $n = 7$ females and males respectively).

2.3 RELATIONSHIP BETWEEN ENDOSYMBIONT INFECTION AND SEX RATIO

Spiders used for investigating the prevalence of the endosymbiont infections and testing the relationship between endosymbiont presence and sex ratio originated from hand catches carried out in the two populations mentioned earlier. Only juvenile and subadult spiders were used to ensure that females were virgin at the start of the experiment. Spiders were reared under the same conditions as mentioned above. At adulthood, females ($n = 15$ and $n = 19$ for Damvallei and Walenbos respectively) were mated with males of the same population and their offspring ($n = 446$ and $n = 485$ for Damvallei and Walenbos respectively) were reared individually till adulthood to determine offspring sex ratio and survival. Females were stored in ethanol after the production of three egg sacs and afterwards screened for the presence of endosymbionts. If a female died before being stored, daughters were screened for endosymbiont presence. Only a single daughter was inspected if the result was positive and the mother was considered to be infected. In the case of a negative result, two additional daughters were screened and if these results were also negative the mother was considered to be uninfected. Offspring of a few females (number of females: $n = 2$ and $n = 5$ for Damvallei and Walenbos respectively) were reared for a second generation to increase sample size (number of offspring: $n = 74$ and $n = 93$ for Damvallei and Walenbos respectively). Significance of a relationship between endosymbiont infection and sex ratio was tested by means of a generalized linear mixed model (Proc GLIMMIX in SAS v.9.1.2) with endosymbiont presence/absence, population and their interaction as fixed effects. To correct for sex ratio variation among females which may inflate the error degrees of freedom, female was included as a random effect.

2.4 ANTIBIOTICS TREATMENT

To test whether an equal sex ratio can be restored after removal of the endosymbionts, we treated one highly distorted maternal line known to be infected with *Wolbachia*, *Rickettsia* and *Cardinium* with antibiotics. This line originated from the breeding experiment to test the relationship between endosymbiont infection and sex ratio distortion (see Methods section 2.3) and was kept in the lab for 7 generations. At generation 4, two females were mated with an unrelated male and 18 offspring from each female were randomly assigned to three different treatments i.e. (i) untreated (to continue the control line and reared as mentioned above), (ii) tetracycline hydrochloride treatment which eliminates *Wolbachia*, *Rickettsia* and *Cardinium* (0.1%, w/v; 0.002 M), (iii) penicillin treatment which only eliminates *Cardinium* (0.1%, w/v; 0.003 M)(Morimoto *et al*., 2006, Gotoh *et al*., 2007). Antibiotics were applied by moistening the vials permanently with the antibiotics solution. Although tetracycline is bacteriostatic (Baron, 1996) and does not remove the bacteria but renders them inactive, PCR screening of antibiotics treated individuals demonstrated that tetracycline removed *Wolbachia* for about half of the specimens. For the remaining individuals, only faint PCR bands, often out of the expected range of the PCR product size were observed. After reaching adulthood, females of each treatment were mated with unrelated males and sex ratio and survival of the clutches was determined (number of females: n = 39, n = 7 and n = 8 for control, penicillin and tetracycline treatment respectively). Differences in average clutch sex ratio between the treatments were analyzed by means of a generalized linear mixed model (Proc GLIMMIX in SAS v. 9.1.2) with treatment as fixed effect. To account for dependence in sex ratio among clutches, mother (factor DAM) was included as a random effect. Survival was generally high and did not differ between spiders treated with tetracycline (mean ± SE: 0.86 ± 0.06), penicillin (mean ± SE: 0.87 ± 0.07) and control spiders (mean ± SE: 0.92 ± 0.02) (Generalized Linear Mixed Model; treatment effect: $F_{2, 40} = 0.89$; $P = 0.42$), indicating that administering antibiotics did not influence offspring survival.

3. RESULTS

3.1 MATERNAL INHERITANCE OF SEX RATIO VARIATION

Previous studies showed that sex ratio in *O. gibbosus* is significantly female distorted, but varies considerably among clutches (Vanacker, 2004). If this observed sex ratio distortion is caused by maternally inherited endosymbiont bacteria, only daughters should inherit the sex ratio trait. By means of an animal model (see Methods section) applied to our extensive pedigree data, the variance in sex ratio among females that is not attributed to sampling error is decomposed into a maternally inherited part (σ_m^2), which only incorporates this part of the sex ratio variation that is transmitted to their daughters, and a residual part (σ_e^2) that estimates the remaining variation. Possible causes of σ_e^2 to deviate from zero include non-random variation in sex ratio among dams, for instance due to loss of the bacterium, genetic variation in resistance, direct paternal effects or other causes of sex ratio variation.

The average sex ratio equalled 0.34 (95% CI: 0.31 – 0.36) and is hence significantly lower than 0.5, but showed considerable variation among clutches (figure 1). Posterior densities of both variance components show that the largest source of sex ratio variation consists of the maternally inherited part. As this variance is much higher than zero (σ_m^2; mean: 0.64; 95% CI: 0.34 – 1.05), this is evidence that the observed sex ratio variation in this species is strongly maternally inherited. However, a smaller but still highly significant residual sex ratio variation (σ_e^2) was observed (mean: 0.27; 95% CI: 0.12 – 0.47), indicating that not all sex ratio variation in this species is maternally inherited and that other factors of minor magnitude are additionally responsible for the among female variation.

The clutch sex ratio was significantly related with the size of the clutch (weighted Pearson correlation; $r = 0.18$; $P = 0.01$), with the proportion of sons being significantly lower in smaller clutches (figure 1).

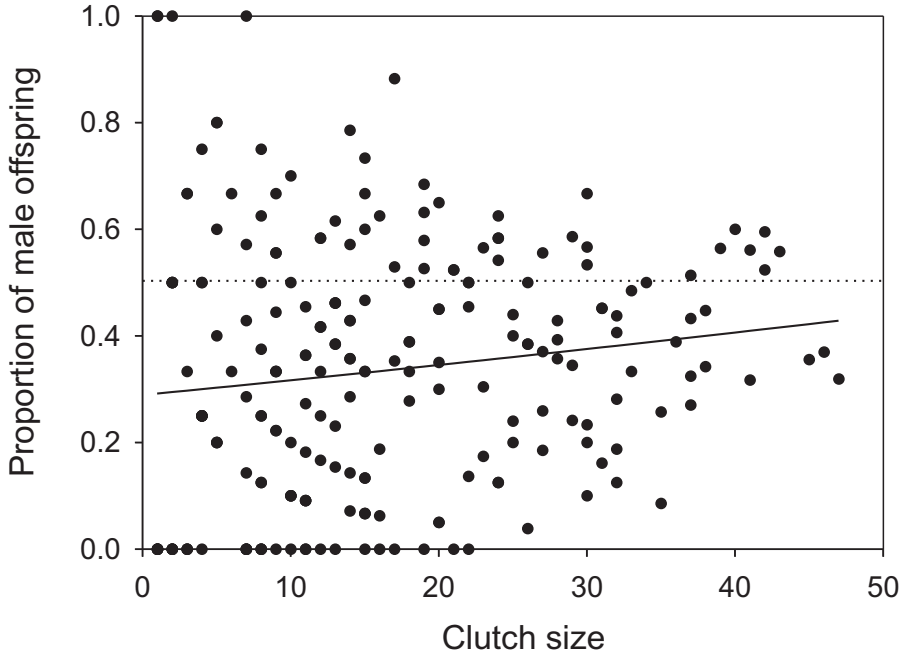


Figure 1. Relationship between proportion of male offspring in a clutch and size of the clutch in the dwarf spider *Oedothorax gibbosus*. Solid line depicts the linear correlation. The dotted line depicts a 50:50 sex ratio and is given for illustrative purposes.

3.2 ENDOSYMBIONT IDENTIFICATION AND PREVALENCE

Single individuals, sampled at two independent populations, tested positive for up to three different endosymbionts capable of altering host reproduction i.e. *Wolbachia*, *Cardinium* and *Rickettsia*. For *Wolbachia*, both sets of primers (i.e. *wsp* and *Wolbachia* specific 16S ribosomal DNA) gave consistent results. Sequences of both genes could be read unambiguously and no among individual variation was observed within the obtained *wsp* and *Wolbachia* specific 16S sequences ([GenBank: HQ286290] and [GenBank: HQ286291] respectively). This suggests that a single *Wolbachia* strain is present in infected individuals. BLAST searches for both genes returned the highest matches with available *Wolbachia* sequences (E-values $<$ 1e-199). For *wsp*, the obtained sequence clustered within supergroup G (See additional file 1: Bayesian inference tree of *Wolbachia wsp* sequences), which according to Rowley et al (2004) primarily comprises of *Wolbachia* endosymbionts of spiders. The recognition of this supergroup as a monophyletic clade has however recently been debated (Baldo and Werren, 2007). Sequences were most closely related with *wsp* sequences found in the spiders *Diaea circumlita* [GenBank: AY486092] and *Hylyphantes graminicola* [GenBank: EU723842], the nematode *Angiostrongylus cantonensis* [GenBank: AY508980] and the mosquito *Malaya genurostris* [GenBank: AY462865] (See additional file 1: Bayesian inference tree of *Wolbachia wsp* sequences). For 16S, the sequence could not be classified unambiguously into one of the supergroups as defined in Lo *et al*. (2002)

and was most closely related to the *Wolbachia* 16S sequence found in the spider *Tetragnatha montana* [GenBank: EU333940] (See additional file 2: Bayesian inference tree of *Wolbachia* 16S sequences). Similarly, sequences for *Rickettsia* and *Cardinium* were easily readable and showed no variation ([GenBank: HQ286289] and [GenBank: HQ286292] respectively). The *citrate* partial sequence of *Rickettsia* clustered within a monophyletic group that consists almost exclusively of *Rickettsia* sequences obtained from other spiders (See additional file 3: Bayesian inference tree of partial *citrate* sequences of *Rickettsia*). The phylogenetic position of the *Cardinium* sequence could not be positioned with high support among one of the other endosymbiont *Cardinium* sequences available at GenBank (See additional file 4: Bayesian inference tree of *Cardinium* 16S sequences). For *Spiroplasma*, only a few faint bands were visible after electrophoresis. However, sequencing and BLAST searches revealed that these were false positives and due to amplification of the bacteria *Acidovorax*.

Approximately half of the individuals were infected with *Wolbachia* with 44% (n = 39) of the females and 57% (n=7) of the males of the Damvallei testing positive. For the Walenbos population 42% (n = 53) of the females and 64% (n = 11) of the males showed *Wolbachia* infection. There was no difference between the sexes in infection frequency for both populations (Fisher's exact test: $P > 0.2$) and no difference in infection frequency between populations ($P = 0.9$). Prevalence of *Rickettsia* and *Cardinium* was fixed in the two investigated populations.

3.3 RELATIONSHIP BETWEEN ENDOSYMBIONT INFECTION AND SEX RATIO

As virtually no variation in infection frequency was observed for *Cardinium* and *Rickettsia*, female infection with these endosymbionts is unlikely to explain the maternal sex ratio variation. However, for *Wolbachia*, infection status of the female had a significant effect on tertiary sex ratio ($F_{1, 36.2} = 6.61$; $P = 0.014$; figure 2), wherein infected females produced a significantly distorted sex ratio (mean ± SE: 0.36 ± 0.04; $t_{37} = -3.70$; $P = 0.0007$) while uninfected females produced a sex ratio that was not significantly different from 0.5 (mean ± SE: 0.47 ± 0.02; $t_{18.1} = -1.81$; $P = 0.086$). The population a female originated from did not have a significant effect on the sex ratio ($F_{1, 36.2} = 0.93$; $P = 0.340$) and the effect of the *Wolbachia* infection was not different between both populations ($F_{1, 36.2} = 0.20$; $P = 0.65$). Within infection groups, no significant variation among females could be detected (estimated variance component: 0.008 ± 0.037; $P = 0.59$).

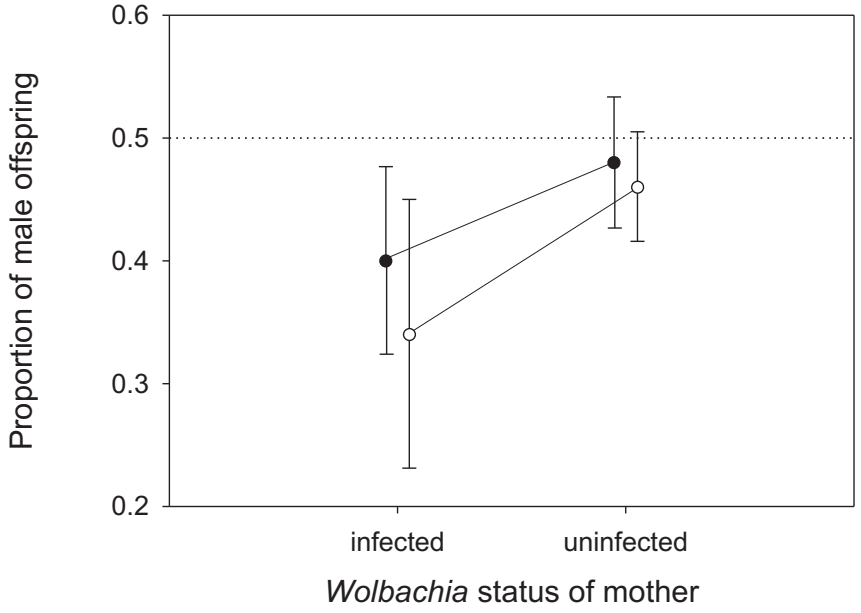


Figure 2. Relationship between average proportion of male offspring in a clutch and *Wolbachia* infection status of the mother that produced the clutch in two different populations. (filled circles = population Damvallei, unfilled circles = population Walenbos). Error bars indicate 95% confidence intervals. The dotted line depicts a 50:50 sex ratio.

3.4 ANTIBIOTICS TREATMENT

Sex ratio produced by females differed significantly among treatments ($F_{2, 34.6} = 4.67$; $P = 0.016$; figure 3). While females of the control group produced a significantly distorted sex ratio (mean $\pm$ SE: 0.25 $\pm$ 0.03; $t_{37.7} = -7.27$; $P < 0.0001$), tetracycline treated females produced an even amount of males and females (mean $\pm$ SE: 0.47$\pm$ 0.08; $t_{28.9} = -0.38$; $P = 0.7$). Although females treated with penicillin also produced a more even sex ratio compared to the control treatment (mean $\pm$ SE: 0.36 $\pm$ 0.08; $t_{40.2} = -1.56$; $P = 0.126$), there was no significant difference compared to the sex ratio of both control and the tetracycline treated females (Tukey post-hoc comparison: $t_{39.9} = -1.35$; $P = 0.38$ and $t_{35} = -0.96$; $P = 0.60$ respectively). Although a single maternal line was used for the experiment, there was still significant variation among females (estimated variance component 0.55 $\pm$ 0.19; $P = 0.002$).

Antibiotic treatment significantly influenced the number of hatched offspring of the first clutch ($F_{2, 39} = 5.14$; $P = 0.010$). There was no significant difference in number of hatched offspring between control spiders (mean ± SE: 16.8 ± 1.6) and penicillin treated spiders (mean ± SE: 9.8 ± 2.2; Tukey post-hoc comparison: $t_{39} = 2.25$; $P = 0.075$). However, a higher number of offspring from females treated with tetracycline (mean ± SE: 24.6 ± 4.5) hatched compared to the penicillin treatment (Tukey post-hoc comparison: $t_{39} = -3.21$; $P = 0.007$) but not compared to the control treatment (Tukey post-hoc comparison: $t_{33.9} = -1.85$; $P = 0.167$).

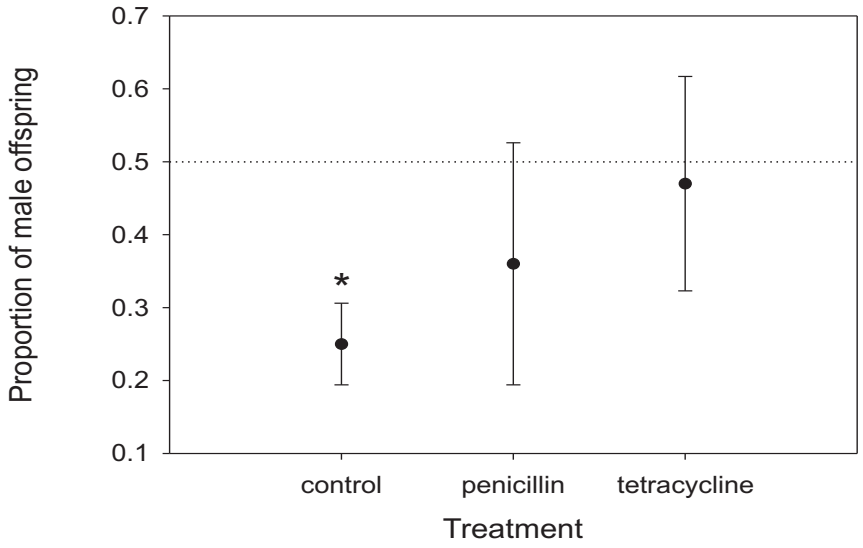


Figure 3: Relationship between average proportion of male offspring in a clutch and antibiotics treatment applied to the mother. Error bars indicate 95% confidence intervals. The dotted line depicts a 50:50 sex ratio. An asterisk indicates proportions significantly different from 0.5.

4. DISCUSSION

Results of our three independent experiments are consistent with the hypothesis that sex ratio variation in the solitary dwarf spider *Oedothorax gibbosus* is caused by reproductive manipulations by endosymbiont bacteria. First, as these bacteria are exclusively transmitted from mother to offspring by means of her eggs, daughters of sex ratio distorted females are expected to produce female distorted clutches as well. In line with this, our pedigree analysis shows that up to 70% of the sex ratio variation among females that is not attributed to sampling error is maternally inherited. Second, PCR-assays showed that *Oedothorax gibbosus* is infected with at least three different endosymbionts known to potentially affect reproductive behavior in arthropods namely: *Wolbachia*, *Rickettsia* and *Cardinium*. Out of these, *Wolbachia* certainly affects sex ratio in our study species; infected females produced a significantly female biased sex ratio compared to the even sex ratio of *Wolbachia* uninfected females. Third, the sex ratio manipulation by endosymbiont bacteria is further confirmed by treating distorted females of bacteria with the antibiotic tetracycline, which restored the production of equal amounts of males and females. Since juvenile survival was in general sufficiently high (>85%) and all female clutches were found for clutches with 100% juvenile survival, it is unlikely that differential juvenile mortality rates of males and females can account for the observed sex ratio bias. The combination of these results unequivocally demonstrates that, among other potentially distorting endosymbionts, *Wolbachia* is able to manipulate sex ratio in spiders. This represents to our knowledge the first clear evidence of a causal relationship between endosymbiont infection and its manipulative effect on host reproduction in spiders.

Several studies confirm the prevalence of several endosymbiont bacteria in the Araneae order, but convincing evidence of their effects on the hosts' reproductive biology is currently lacking. As argued by Weeks *et al.* (2002), the establishment of multiple lines of evidence are a prerequisite to confirm the manipulative effect exhibited by these microorganisms. Previously, one such connection has been suggested by Gunnarsson *et al.* (2009) who found an effect of female size on female post-copulatory position, which in turn affects brood sex ratio in *Pityohyphantes phrygianus*. This effect is altered by an interaction of the *Wolbachia* infection status of the male and infected female size which makes the observed effect indirect and subject to female control. Duron *et al.* (2008a) suggested manipulative effects by reporting a sex biased prevalence of *Wolbachia* in the spiders *Tetragnatha montana* and *Meta mengei*. It remains however unknown whether sex ratio distortion is present in those species.

Identifying the phylogenetic position of the *Wolbachia* strain of *Oedothorax gibbosus* might help to further explore the incidence of reproductive manipulations by *Wolbachia* in spiders. Based on the *wsp* sequences, the strain present in our study species is situated within supergroup G, previously reported by Rowley *et al.* (2004) to be spider specific and has highest similarities with the strains found in the spider genera *Diaea* and *Dysdera*. These genera are known to exhibit primary sex ratio distortion (*Diaea* (Rowell and Main, 1992)) and parthenogenesis induction (*Dysdera* (Deeleman-Reinhold, 1986)). The classification of these sequences into a monophyletic distinct clade G is however not well supported, not only as it is merely based on a single gene, but additionally because it has been suggested to represent a recombinant of the supergroup A and B *wsp* sequences (Baldo and Werren, 2007). For 16S, unambiguous classification into one of

the existing supergroups could not be supported, but it is most closely related to the sequences found in the spider *Tetragnatha montana*. Remarkably, this was also one of the two species out of the 26 spiders species tested by Duron *et al*. (2008a) where a manipulative effect of *Wolbachia* was suggested based on a higher prevalence in females compared to males.

Notwithstanding, whilst *Wolbachia* obviously plays an important role in the reproductive biology of *O. gibbosus*, our findings also point out that there is no simple one-to-one relationship between infection status and sex ratio distortion. First, if *Wolbachia* infection status alone was responsible for sex ratio variation, all among female variation would be expected to be inherited maternally. Our pedigree analysis in contrast revealed that a smaller though significant part of the among female variation is not strictly inherited from mother to daughters. Although the most straightforward explanation would be an imperfect transmission of the bacterium towards offspring, PCR screening demonstrated for a subset of the data that infected females ($n = 4$) only produced infected offspring ($n = 47$), indicating very high transmission efficiencies. Paternal effects, whether caused by meiotic drive, i.e. the unequal production of male and female producing sperm (Jaenike, 2001), or suppression of the manipulative effect by nuclear genes (Hornett *et al.*, 2006, Majerus and Majerus, 2010) are therefore more plausible mechanisms. Second, paternal effects are also suggested from the antibiotics treatment experiment; although a single maternal line was used there is still significant variation among females within the control treatment, which could be explained by the fact that females were mated with different males. Variation in *Wolbachia* titer, potentially induced by *Wolbachia* specific bacteriophages, may also substantially affect the expression of the manipulative effect (Bordenstein *et al*., 2006). It remains however at present less understood which factors determine *Wolbachia* concentrations in arthropods (Serbus *et al*., 2008). Third, a relative large proportion of males are still infected with *Wolbachia*, indicating an imperfect manipulative effect or the presence of resistance genes in both populations. *Wolbachia* infection of field captured individuals even suggests a higher prevalence in males compared to females, although this can be most probably attributed to sampling error. Indeed, given that the observed prevalence averaged over both populations equals 0.45, and that the observed sex ratio of infected females averages 0.36, the estimated proportion of individuals that are male and infected (i.e. males that originate from infected females) equals $(0.45)*(0.36) = 0.162$. Likewise, the proportion of individuals that are male and uninfected (i.e. males that originate from uninfected females) equals $(0.55)*(0.5) = 0.275$. Hence, the estimated proportion of infected males equals $0.162/(0.162 + 0.275) = 0.37$. An exact binomial test however reveals that the observation of 11 infected males out of 18 males tested is not sufficient to reject the hypothesis that only 37% of the males are infected ($P = 0.07$).

Multiple species of endosymbionts were found to infect the same individual, which creates the opportunity for interaction effects to occur. Our use of several antibiotics that target different endosymbiont species allows to investigate the potential occurrence of such interaction effects. However, penicillin treated females, expected to only target *Cardinium*, did not result in a significantly different offspring sex ratio compared to the control and tetracycline treatment. Therefore no indications of the potential additional effect of *Cardinium* on the sex ratio could be observed.

It is known that *Wolbachia* can induce several reproductive alterations like parthenogenesis induction, male-killing and feminization (Stouthamer *et al*., 1999, Werren *et al*., 2008, Engelstädter and Hurst, 2009). Parthenogenesis induction is highly unlikely to occur since no offspring were produced by unmated females (pers. obs.). A female biased sex ratio can further be caused both by male killing and feminization. If male killing is present, the number of hatched offspring from infected females is typically half of that from uninfected females since male embryos are selectively killed. Feminization, however, converts genetic males into phenotypic females which results in an equal number of hatching offspring.

Based on our pedigree data, we found a significant positive correlation between clutch size and proportion of sons in each clutch, wherein smaller clutches were significantly more female biased. This points into the direction of killing of male embryos as the most likely manipulating mechanism. Nevertheless, more exclusive evidence could have been obtained from the antibiotics treatment. Although the higher hatching rate of tetracycline treated individuals approached twice the clutch size of control females, which is congruent with the expectations under male killing, this difference was not significant. The difficulty to clearly infer about the possible manipulating mechanism in our study species is most likely attributed to the large variance in clutch size (see figure 1). Relationships between clutch size and sex ratio could therefore only be observed for the extensive pedigree data set.

Recognizing the effect of microorganisms on the reproductive biology of spiders is of high importance to further understand the mechanisms that cause the pronounced diversity of reproductive phenotypes observed in this arthropod order. At least for our male dimorphic study species (Vanacker *et al*., 2004), the sex ratio distorting effect of *Wolbachia* could be involved in the stable coexistence of both distinct male phenotypes through its influence on sexual selection, as suggested by theoretical (Wade *et al*., 2003) as well as empirical (Shuster and Sassaman, 1997) investigations. In addition, our findings of an important role of endosymbionts on sex ratio distortion might provide a useful framework to decipher the mechanisms that cause parthenogenesis in spiders (Camacho, 1994) and the facilitation of adaptive sex ratio adjustments that are commonly observed in social spiders to reduce male mate competition (Avilés *et al*., 1999, Avilès *et al*., 2000).

CONCLUSIONS

In this study we adopted a threefold strategy to corroborate the role of endosymbiont bacteria in causing a female distorted sex ratio in the solitary dwarf spider *Oedothorax gibbosus*. (i) Pedigree analysis confirms that the sex ratio trait is primarily maternally inherited, (ii) PCR-assays show that individuals are infected with up to three endosymbionts known to cause reproductive alterations in arthropods, i.e. *Wolbachia*, *Rickettsia* and *Cardinium* and that females infected with *Wolbachia* produce significantly more females than males compared to uninfected females. (iii) Antibiotic curing of *Wolbachia* infected females restores the production of an equal amount of males and females. This is the first direct evidence of endosymbiont interference in reproductive characteristics in the Araneae order. These findings can have major implications on understanding the mechanism causing the variety of reproductive phenotypes present in spider species.

AUTHORS' CONTRIBUTION

Bram Vanthournout and Frederik Hendrickx conceived and designed the study and wrote the manuscript. Bram Vanthournout, Frederik Hendrickx and Janne Swaegers analyzed the data and performed the breeding experiments and molecular analyses.

ACKNOWLEDGEMENTS

We are indebted to Jean-Pierre Maelfait for initiating the research on this most intriguing study organism. He unfortunately deceased shortly before the start of this particular study. With him, we lost an outstanding scientist and colleague. Additional pedigree data were kindly provided by Danny Vanacker. Andy Vierstraete performed part of the sequencing analysis. Viki Vandomme provided help in rearing collembolans, preparing fruit fly media and performing hand catches. BV received a PhD scholarship from IWT (grant n° 73344). Additional financial support was achieved from the Belgian Science Policy (BELSPO, research project MO/ 36 / 025).

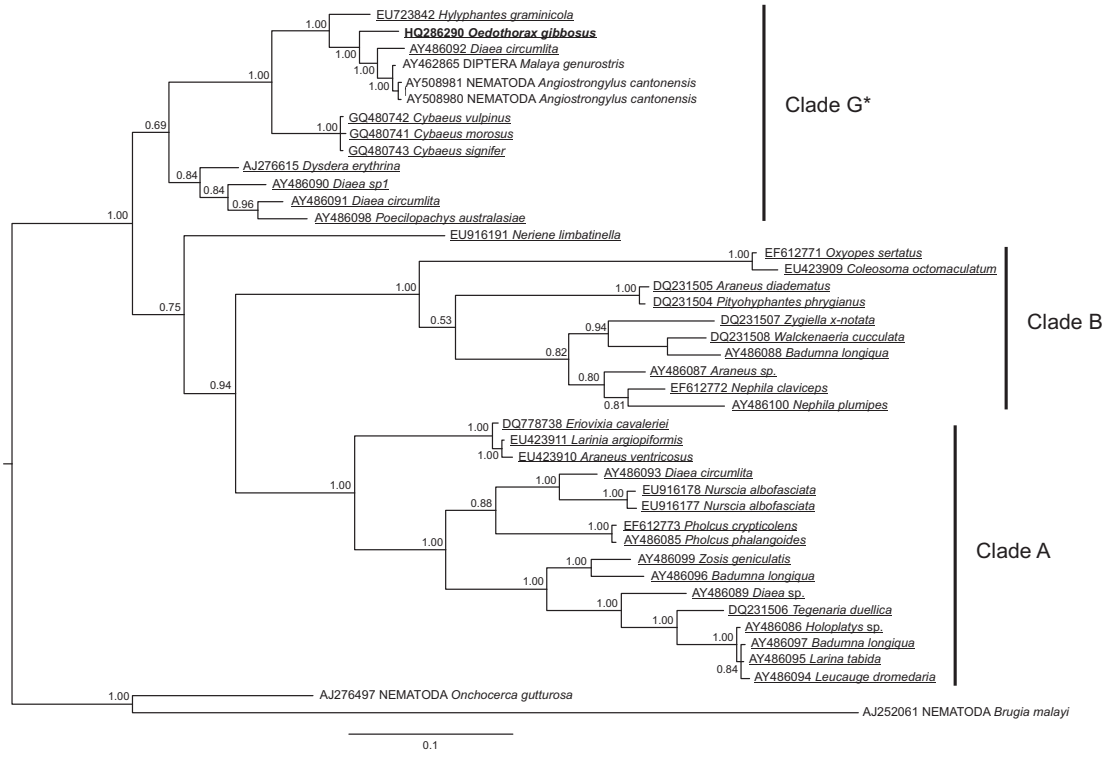
5. REFERENCES

- AVILÈS, L. 1986. Sex-ratio bias and possible group selection in the social spider *Anelosimus eximius*. *The American Naturalist*, 128, 1-12.
- AVILÈS, L., MCCORMACK, J., CUTTER, A. & BUKOWSKI, T. 2000. Precise, highly female-biased sex ratios in a social spider. *Proc. R. Soc. Lond. B*, 267, 1445-1449.
- AVILÉS, L., VARAS, C. & DYRESON, E. 1999. Does the African social spider *Stegodyphus dumicola* control the sex of individual offspring. *Behav. Ecol. Sociobiol.*, 46, 237-243.
- BALDO, L. & WERREN, J. H. 2007. Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. *Current Microbiology*, 55, 81-87.
- BARON, S. 1996. *Medical Microbiology*, Galveston, Texas, University of Texas Medical Branch.
- BORDENSTEIN, S. R., MARSHALL, M. L., FRY, A. J., KIM, U. & WERNEGREEN, J. J. 2006. The tripartite associations between bacteriophage, *Wolbachia* and arthropods. *PLOS Pathogens*, 2, e43.
- BRAIG, H. R., ZHOU, W. G., DOBSON, S. L. & O'NEILL, S. L. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *Journal of Bacteriology*, 180, 2373-2378.
- CAMACHO, J. P. M. 1994. Female-biased sex ratio in spiders caused by parthenogenesis. *Hereditas*, 120, 183-185.
- CHARLAT, S., HURST, G. D. D. & MERÇOT, H. 2003. Evolutionary consequences of *Wolbachia* infections. *Trends in Genetics*, 19, 217-222.
- DAMGAARD, L. H. 2007. How to use Winbugs to draw inferences in animal models. *J. Anim. Sci.*, 85, 1363-1368.
- DAVIS, M. J., YING, Z., BRUNNER, B. R., PANTOJA, A. & FERWERDA, F. H. 1998. Rickettsial relative associated with Papaya bunchy top disease. *Curr. Microbiol.*, 36, 80-84.
- DEELEMAN-REINHOLD, C. L. Year. *Dysdera hungarica* Kulczynski - a case of parthenogenesis? In: Actas X Congreso Internacional de Arachnologica, 1986 1986 Jaca, Spain. Impreso por Imprenta Juvenil, Barcelona.
- DURON, O., BOUCHON, D., BOUTIN, S., BELLAMY, L., ZHOU, L., ENGELSTÄDTER, J. & HURST, G. D. 2008a. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology*, 6:27.
- DURON, O., HURST, G. D. D., HORNETT, E. A., JOSLING, J. A. & ENGELSTÄDTER, J. 2008b. High incidence of the maternally inherited bacterium *Cardinium* in spiders. *Mol. Ecol.*, 1-11.
- EDWARDS, R. L., EDWARDS, E. H. & EDWARDS, A. D. 2003. Observations of *Theotima Minutissimus* (Araneae, Ochyroceratidae), a Parthenogenetic Spider. *Journal of Arachnology*, 31, 274-277.
- ENGELSTÄDTER, J. & HURST, G. D. D. 2009. The ecology and evolution of microbes that manipulate host reproduction. *Annu. Rev. Ecol. Evol. Syst.*, 40, 127-149.
- GIRIBET, G., CARRANZA, S., BAGUÑA, J., RIUTORT, M. & RIBERA, C. 1996. First molecular evidence for the existence of a Tartigrada+Arthropoda clade. *Mol. Biol. Evol.*, 13, 76-84.
- GOODACRE, S., MARTIN, O. Y., THOMAS, C. F. G. & HEWITT, G. M. 2006. *Wolbachia* and other endosymbiont infections in spiders. *Mol. Ecol.*, 15, 517-527.
- GOTOH, T., NODA, H. & ITO, S. 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. *Heredity*, 98, 13-20.

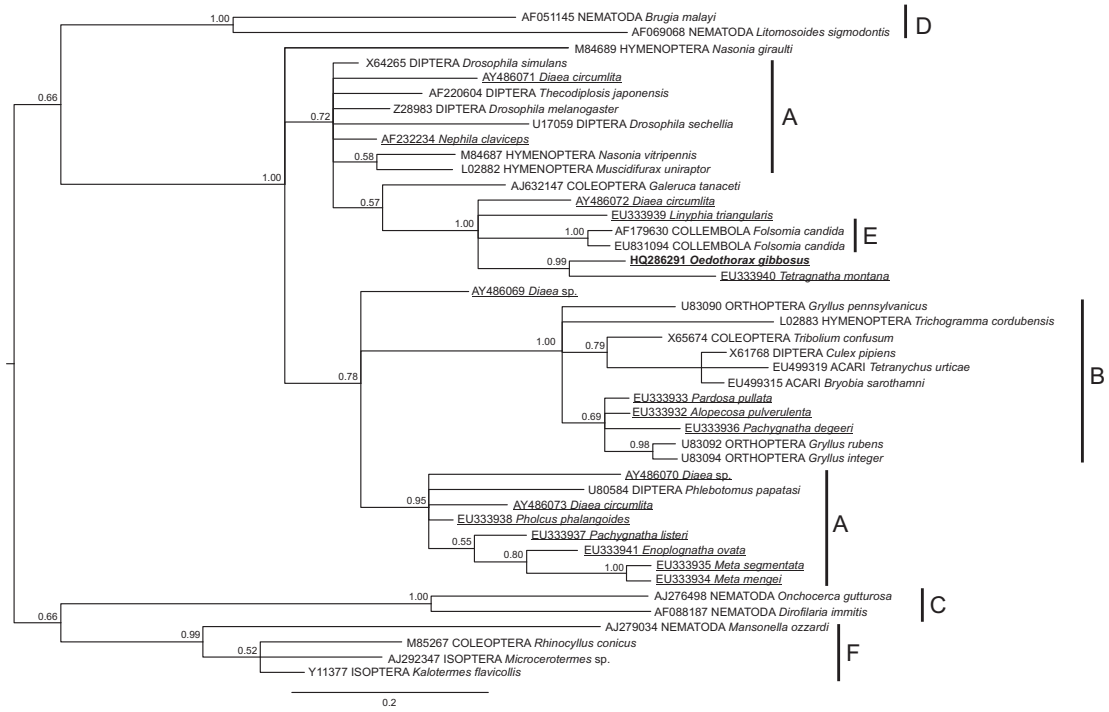
- GUNNARSSON, B., GOODACRE, S. L. & HEWITT, G. M. 2009. Sex ratio, mating behaviour and *Wolbachia* infections in a sheetweb spider. *Biological Journal of the Linnean Society*, 98, 181-186.
- GUNNARSSON, B., UHL, G. & WALLIN, K. J. 2004. Variable female mating positions and offspring sex ratio in the spider *Pityohyphantes phrygianus* (Araneae: Linyphiidae). *Journal of Insect Behavior*, 17, 129-144.
- HILGENBOECKER, K., HAMMERSTEIN, P., SCHLATTMANN, P., TELSCHOW, A. & WERREN, J. H. 2008. How many species are infected with *Wolbachia*?- a statistical analysis of current data. *FEMS Microbiol. Lett.*, 281, 215-220.
- HORNETT, E. A., CHARLAT, S., DUPLOUY, A. M. R., DAVIES, N., RODERICK, G. K., WEDELL, N. & HURST, G. D. D. 2006. Evolution of male-killer suppresion in a natural population. *Plos Biology*, 4, 1643-1648.
- HUELSENBECK, J. P. & RONQUIST, F. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17, 754-755.
- JAENIKE, J. 2001. Sex Chromosome Meiotic Drive. *Annu. Rev. Ecol. Syst*, 49, 25-49.
- JEYAPRAKASH, A. & HOY, M. A. 2000. Long PCR improves *wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Molecular Biology*, 9, 393-405.
- LO, N., CASIRAGHI, M., SALATI, E., BAZZOCHI, C. & BANDI, C. 2002. How many *Wolbachia* supergroups exist? *Mol. Biol. Evol.*, 19, 341-346.
- LYNCH, M. & WALSH, B. 1998. *Genetics and analysis of quantitative traits.*, Sunderland, MA, Sinauer Associates.
- MAJERUS, M. E. N., HINRICH, J., SCHULENBURG, G. V. D. & ZAKHAROV, I. A. 2000. Multiple Causes of Male-Killing in a Single Sample of the Two-Spot Ladybird, *Adalia Bipunctata* (Coleoptera : Coccinellidae) From Moscow. *Heredity*, 84, 605-609.
- MAJERUS, T. M. O. & MAJERUS, M. E. N. 2010. Intergenomic arms races: detection of a nuclear rescue gene of male-killing in a ladybird. *PLOS Pathogens*, 6, e1000987.
- MAJERUS, T. M. O., VON DER SCHULENBURG, J. H. G., MAJERUS, M. E. N. & HURST, G. D. D. 1999. Molecular identification of a male-killing agent in the ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). *Ins. Mol. Biol.*, 8, 551-555.
- MARTIN, O. Y. & GOODACRE, S. L. 2009. Widespread infections by the bacterial endosymbiont *Cardinium* in arachnids. *J. arachnol*, 37, 106-108.
- MORIMOTO, S., KURTTI, T. J. & NODA, H. 2006. In vitro cultivation and antibiotic susceptibility of a *Cytophaga*-like intracellular symbiote isolated from the tick *Ixodes scapularis*. *Curr. Microbiol.*, 52, 324-329.
- O'NEILL, S. L., GIORDANO, R., COLBERT, A. M. E., KARR, T., L & ROBERTSON, H. M. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Nat. Acad. Sci.*, 89, 2699-2702.
- OH, H. W., KIM, M. G., SHIN, S. W., BAE, K. S., AHN, Y. J. & PARK, H.-Y. 2000. Ultrastructural and molecular identification of a *Wolbachia* endosymbiont in a spider, *Nephila clavata*. *Insect Molecular Biology*, 9, 539-543.
- RAMBAUT, A. J. & DRUMMOND, A. 2007. Tracer. 1.4 ed.: http://tree.bio.ed.ac.uk/software/tracer/.
- ROWELL, D. M. & MAIN, B. Y. 1992. Sex ratio in the social spider *Diaea socialis* (Araneae: Thomisidae). *J. arachnol*, 20, 200-206.

- ROWLEY, S. M., RAVEN, R. J. & MCGRAW, E. A. 2004. *Wolbachia pipientis* in Australian Spiders. *Current Microbiology*, 49, 208-214.
- SERBUS, L. R., CASPER-LINDLEY, C., LANDMANN, F. & SULLIVAN, W. 2008. The genetics and cell biology of *Wolbachia*-host interactions. *Annu. Rev. Genet.*, 42, 683-707.
- SHUSTER, S. M. & SASSAMAN, C. 1997. Genetic interaction between male mating strategy and sex ratio in a marine isopod. *Nature*, 388, 373-376.
- STOUTHAMER, R., BREEUWER, J. A. J. & HURST, D. D. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Micribiol.*, 53, 71-102.
- TAMURA, K., DUDLEY, J., NEI, M. & KUMAR, S. 2007. Mega4: Molecular Evolutionary Genetics Analysis (Mega) Software Version 4.0. *Molecular Biology and Evolution*, 24, 1596-1599.
- VANACKER, D. 2004. *Morphology, function and evolution of the male cephalic structures of the dwarf spider genus Oedothorax*. PhD, Ghent University.
- VANACKER, D., HENDRICKX, F., MAES, L., VERRAES, P. & MAELFAIT, J.-P. 2004. Can multiple mating compensate for slower development and shorter adult life in a male dimorphic dwarf spider? *Biol. J. Linn. Soc.*, 82, 269-273.
- WADE, M. J., SHUSTER, S. M. & DEMUTH, J. P. 2003. Sexual selection favors female-biased sex ratios: the balance between the opposing forces of sex-ratio selection and sexual selection. *Am. Nat.*, 162, 403-414.
- WEEKS, A. R., REYNOLDS, T. K. & HOFFMANN, A. A. 2002. *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated? *Trends Ecol. Evol.*, 17, 257-262.
- WERREN, J. H., BALDO, L. & CLARK, M. E. 2008. *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews: Microbiology*, 6, 741-751.
- WERREN, J. H. & BEUKEBOOM, L. W. 1998. Sex determination, sex ratios, and genetic conflict. *Annu. Rev. Ecol. Syst.*, 29, 233-261.

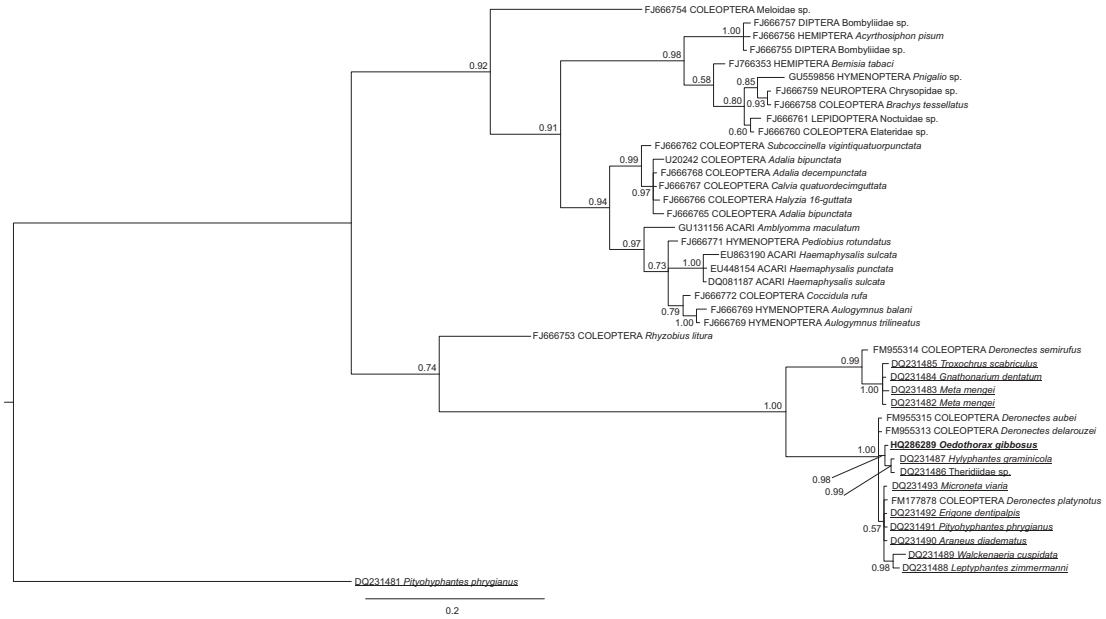
6. ADDITIONAL FILES



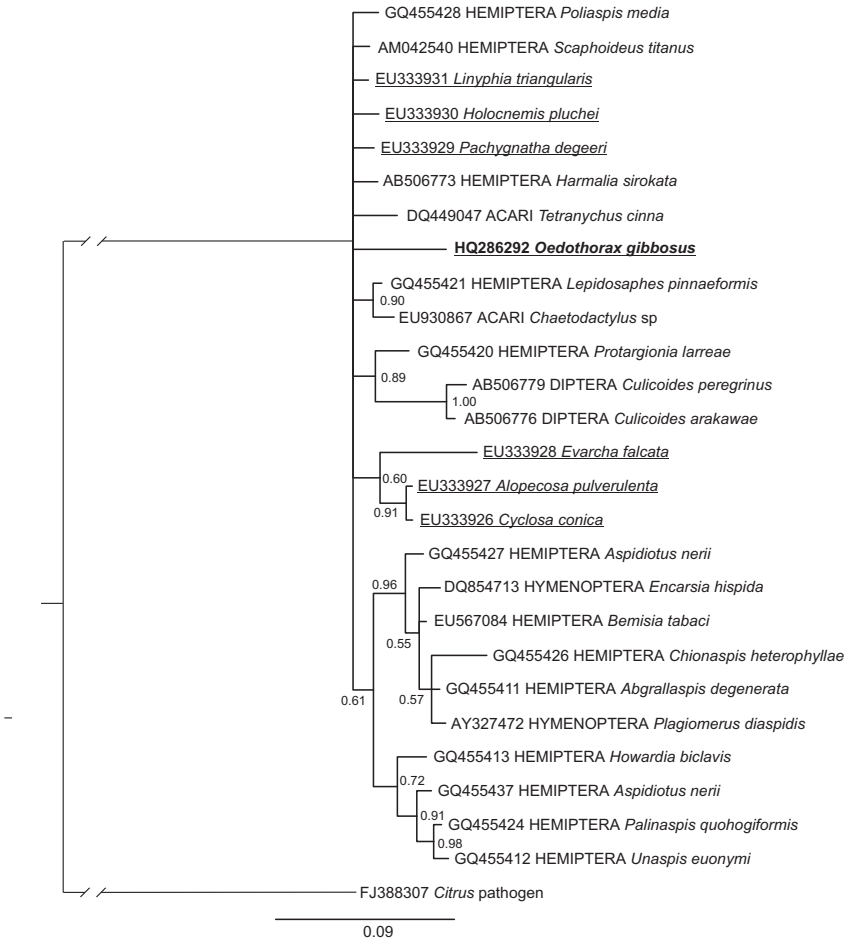
Additional file 1. Phylogenetic position of *Wolbachia* wsp sequence of *Oedothorax gibbosus* [GenBank:HQ286290]. Tree was constructed by Bayesian tree searching as implemented in MrBayes (Huelsenbeck and Ronquist, 2001) on a subset of *Wolbachia wsp* sequences available at GenBank, with indication of the major *Wolbachia* supergroups. Node values represent posterior probabilities of the clades. Genbank accession numbers are given in front of the species name. Sequences that do not originate from spider hosts are preceded with the taxonomic group to which the host species belongs, spider hosts are underlined. *Oedothorax gibbosus* is shown in bold.



Additional file 2. Phylogenetic position of *Wolbachia* 16S rDNA sequence of *Oedothorax gibbosus* [GenBank:HQ286291]. Tree was constructed by Bayesian tree searching as implemented in MrBayes (Huelsenbeck and Ronquist, 2001) on a subset of *Wolbachia* 16S rDNA sequences available at GenBank, with indication of the major *Wolbachia* supergroups. Node values represent posterior probabilities of the clades. Genbank accession numbers are given in front of the species name. Sequences that do not originate from spider hosts are preceded with the taxonomic group to which the host species belongs, spider hosts are underlined. *Oedothorax gibbosus* is shown in bold.



Additional file 3. Phylogenetic position of *Rickettsia* (partial *citrate* sequence) endosymbiont of *Oedothorax gibbosus* [GenBank:HQ286289]. Tree was constructed by Bayesian tree searching as implemented in MrBayes (Huelsenbeck and Ronquist, 2001) on a subset of *Rickettsia* sequences available at GenBank. Node values represent posterior probabilities of the clades. Genbank accession numbers are given in front of the species name. Sequences that do not originate from spider hosts are preceded with the taxonomic group to which the host species belongs, spider hosts are underlined. *Oedothorax gibbosus* is shown in bold.



Additional file 4. Phylogenetic position of *Cardinium* (16S rRNA gene) endosymbiont of *Oedothorax gibbosus* [GenBank:HQ286292]. Tree was constructed by Bayesian tree searching as implemented in MrBayes (Huelsenbeck and Ronquist, 2001Huelsenbeck and Ronquist, 2001) on a subset of *Cardinium* sequences available at GenBank. Node values represent posterior probabilities of the clades. Genbank accession numbers are given in front of the species name. Sequences that do not originate from spider hosts are preceded with the taxonomic group to which the host species belongs, spider hosts are underlined. *Oedothorax gibbosus* is shown in bold.

"Where there is one, there are many."

Unknown

CHAPTER 2

A SECOND MALE-KILLING CYTOPLASMIC FACTOR IN THE DWARF SPIDER *OEDOTHORAX GIBBOSUS*

BRAM VANTHOURNOUT [1]

VIKI VANDOMME [1]

FREDERIK HENDRICKX [1,2]

[1] Terrestrial Ecology Unit, Department of Biology, Ghent University, Ledeganckstraat 35, 9000 Ghent, Belgium

[2] Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium

ABSTRACT

Endosymbiont bacteria can bring about profound changes in their host biology due to the reproductive alterations they induce resulting in the increase of their own transmission. Screening studies show that a high number of arthropod species are infected with endosymbionts and that one host species can show infection with multiple species of endosymbiont bacteria. Although the knowledge of the effects of such endosymbiont infection is currently lagging behind, a few well studied cases reveal distinct reproductive effects of multiple endosymbiont species within one host species. However, this persistence of multiple species of endosymbionts is still poorly understood as theoretical approaches show that stable coexistence is possible only under specific conditions. The dwarf spider *Oedothorax gibbosus* has previously been shown to be infected with *Cardinium*, *Rickettsia* and *Wolbachia*, with the latter causing a female biased sex ratio of approximately 30% males by male-killing. In this study we show that *Wolbachia* is unlikely to be the only sex ratio distorting agent in this species as few females, uninfected with *Wolbachia*, produced clutches with an even lower proportion of males. Given that this sex ratio trait is inherited only through daughters, and that tetracycline treatment restored sex ratio towards the production of an equal proportion of males and females, infection with a second male-killing endosymbiont is suggested. As current attempts to identify this endosymbiont failed, we cannot distinguish if this is caused by one of the present endosymbiont bacteria, in particular *Rickettsia*, or a yet unidentified endosymbiont that is present in very low numbers in infected females. Furthermore, an additional endosymbiont was found to infect individuals of *Oedothorax gibbosus* as molecular analysis revealed the presence of *Rhabdochlamydia*. Moreover, this endosymbiont infection seems to be population dependent as it was only detected in one of the two populations sampled.

1. INTRODUCTION

Endosymbiont bacteria that affect their hosts reproduction are considered important actors in shaping their hosts reproductive biology and as such may impact evolutionary processes by the phenotypic effects they impose (Hurst and Werren, 2001, Charlat *et al*., 2003, Cordaux *et al*., 2011). Due to their almost exclusive maternal inheritance, they primarily alter reproductive strategies resulting in a higher proportion of infected females in the population. These are male-killing, feminization, parthenogenesis induction and cytoplasmic incompatibility (CI) (Werren, 1997, Stouthamer *et al*., 1999, Bandi *et al*., 2001, Werren *et al*., 2008, Engelstadter and Hurst, 2009).

The five bacterial genera of endosymbionts that infect arthropods and that are known to exhibit such phenotypes are *Wolbachia*, *Rickettsia*, *Cardinium*, *Spiroplasma* and *Arsenophonus* (Bandi *et al*., 2001, Moran *et al*., 2008, Engelstadter and Hurst, 2009). Traditionally, the reproductive effects of *Wolbachia* endosymbionts have been most frequently studied, however, the discovery of other endosymbiont species causing similar reproductive phenotypes led to the understanding that a particular phenotype can be caused by different species of endosymbiont bacteria (Weeks *et al*., 2002). In line with this, screening studies show that species, populations and even individuals are often infected with multiple species or strains of endosymbiont bacteria (Majerus, 2006). This not only led to the increasing interest in the individual effects of each endosymbiont species, but moreover in the cause of potential interactions within this microbial community. Although in general the knowledge of the effects of endosymbiont infection through empirical verification is lagging behind, some systems are studied more in detail, revealing the effects of multiple endosymbiont species or strains in particular species (Charlat *et al*., 2006), populations (Hurst *et al*., 1999, Majerus *et al*., 2000, Jiggins *et al*., 2001) and even individuals (Rousset and Solignac, 1995). Especially for the male-killing strategy, several studies showed that multiple male-killers can coexist within the same species. In ladybirds, wherein a high number of species are infected with male-killing endosymbionts, multiple infections cause a similar male-killing phenotype (Majerus, 2006). In *Adalia bipunctata* male-killing effects have been assigned to endosymbionts of the genera *Rickettsia*, *Spiroplasma* and two strains of *Wolbachia* (Hurst et al., 1999, Majerus et al., 2000). Two other species, *Harmonia axyridis* and *Cheilomenes sexmaculata*, are suspected to exhibit multiple infections (Majerus, 2006). In the butterfly species *Acraea encedon* two strains of male-killing *Wolbachia* are found (Jiggins *et al*., 2001) and in the butterfly *Hypolimnas bolina* a male-killing Wolbachia is present as well as a *Wolbachia* strain inducing almost complete CI (Charlat *et al*., 2006). It is argued that certain host life history traits play an important role in the probability of acquisition and invasion of male-killers (Hurst, 1991, Hurst and Majerus, 1993, Majerus and Hurst, 1997, Elnagdy *et al*., 2011). For example, host species with high levels of intraspecific competition for resources and sibling cannibalism are expected to be prone to invasion by a male-killer as the killing and consuming of dead brothers results in decreased resource competition and a direct nutritional advantage for infected daughters (but see Jiggins *et al*., 2000).

Theoretical approaches, however, reveal that two male-killers can only stably coexist in one population under certain specific conditions. Two male-killers with different transmission frequencies can persist in one population when spatially structured populations are considered

(Groenenboom and Hogeweg, 2002) and in the presence of sibmating with high male mating capacity (Dannowski *et al*., 2009). Also, a male-killing endosymbiont with low transmission rate can invade and persist in a population harbouring a male-killer with high transmission if host resistance genes evolved against the stronger male-killer (Randerson *et al*., 2000). Recently it has been shown that individuals of the dwarf spider *Oedothorax gibbosus* are infected with a male-killing *Wolbachia* endosymbiont with a prevalence of approximately 45% (Vanthournout *et al*., 2011). Two other endosymbiont genera , i.e. *Rickettsia* and *Cardinium*, were found to be fixed in the populations investigated. Pedigree analysis revealed that not all sex ratio variation could be explained by a strict maternal inheritance pattern. This suggests the presence of additional factors influencing the sex ratio, such as the action of paternal suppressor genes. Additionally, the high frequency (64%, n = 11) of adult males infected with *Wolbachia* also indicates the presence of a suppressing effect on the male-killing phenotype. According to theoretical and ecological prerequisites, the suspected presence of a suppressing factor and the similarities with life history traits of ladybirds and butterflies, such as egg laying grouped in egg sacs, the occurrence of sibling cannibalism and high levels of juvenile mortality, indicate that *Oedothorax gibbosus* could be highly liable to invasion by male-killers. Therefore, we investigated the hypothesis that this species has been indeed invaded by multiple male-killers by resampling populations for which *Wolbachia* effects have been demonstrated and determining if endosymbiont induced distorted sex ratios are present in the absence of *Wolbachia*.

2. MATERIAL AND METHODS

2.1 FIELD COLLECTION AND BREEDING DESIGN

Subadult and adult female spiders were collected by hand in two populations Damvallei and Walenbos in 2010 (Vanthournout *et al*., 2011) and 2011. Sex ratio of their clutches was determined in the lab and subsequently tested for the presence of *Wolbachia* (see 2.2), which is known to distort sex ratio in this species (Vanthournout *et al*., 2011). Females were reared individually in the lab under standardized conditions as described in Vanthournout *et al*. (2011). Upon reaching adulthood, a single male was placed in the vial of a female for 24 h to allow copulation. Females were first allowed to lay egg sacs in the vial and were afterwards stored in 97% ethanol. Offspring were reared individually and their sex was assessed at adulthood using a stereomicroscope to determine tertiary sex ratio (number of adult males / number of total adult offspring). Offspring of one Walenbos female (W-01), uninfected with *Wolbachia* and producing a significantly distorted sex ratio, was further mated with unrelated males. This matriline (Wol-) was kept in the lab for six generations to determine the inheritance pattern of the sex ratio bias (number of crosses = 30; total number of offspring = 611). The effect of this sex ratio distorting factor was compared to a *Wolbachia* infected matriline (Wol+), originating from the same Walenbos population and reared for an identical number of generations in the lab (number of crosses = 31; total number of offspring = 797). Differences between the two matrilines were analyzed using a generalized linear mixed model with matriline as a fixed effect (proc GLIMMIX in SAS v. 9.1.2). Dependence between offspring sex ratios from egg sacs originating from the same male was tested and accounted for by including female ID, nested within male, as a random effect.

To investigate the underlying mechanism that causes sex ratio distortion in the Wol- matriline, we related clutch sex ratio to clutch size by means of a Pearson correlation weighted for number of adult offspring. Under male-killing, clutch size is expected to be lowered in distorted clutches due to the killing of male offspring. However, under feminization of genetic males into phenotypic females, clutch size is expected to be unaltered in highly distorted clutches.

2.2 ENDOSYMBIONT SCREENING

Three different molecular techniques were used to characterize the endosymbionts in this species. First, PCR assays with endosymbiont specific primers were performed to determine the presence of seven genera of arthropod infecting endosymbionts of which five are known to cause reproductive alterations. As this technique is limited by the primer specificity, we next amplified 16S rRNA with general eubacterial primer based techniques and investigated for variation in PCR amplicons by means of cloning and denaturating gel gradient electrophoresis (DGGE).

2.2.1 ENDOSYMBIONT SPECIFIC POLYMERASE CHAIN REACTION (PCR) ASSAYS

Whole spiders were used for DNA extraction using the Nucleospin Tissue kit (©Machery Nagel) following the manufacturers recommended protocol. The following primers were used: (i) 16Swolb99F and 16Swolb99R (Oneill *et al*., 1992) to amplify the 16S ribosomal RNA gene of *Wolbachia*; (ii) *WSP*81F and *WSP*691R (Braig *et al.*, 1998) amplifying a part of the cell surface protein coding *wsp* gene of *Wolbachia* (iii) RICS741F and RICT1197R (Davis *et al*., 1998, Majerus *et al*., 2000), which amplify a part of the *citrate* gene of *Rickettsia*; (iv) CLO -f1 and CLO-r1 (Gotoh *et al*., 2007) to selectively amplify a part of the 16S rRNA gene of *Cardinium* (v) SP-ITS-J04 and SP-ITS-N55 (Majerus *et al*., 1999) to selectively amplify the spacer region between the 16S and 23S rRNA genes of *Spiroplasma ixodetis*, (vi) ArsF, ArsF3/ArsR2 to amplify a part of the 16S rDNA of *Arsenophonus nasoniae* (Duron *et al*., 2008), (vii) the general eubacterial primer 27F (Weisburg *et al*., 1991) and PASScmp (Lundgren *et al*., 2007) which amplifies a part of the 16S rDNA of *Serratia symbiotica* (Fukatsu *et al*., 2000) and (viii) F1 and R1 which amplify a part of the type specific antigen of *Orientia* (Takahashi *et al*., 1997).

Results of the cloning assay (see 3.3) revealed the presence of *Rhabdochlamydia* in individuals of Walenbos. To investigate the prevalence of this endosymbiont in the Walenbos en Damvallei population, primers Rhab16S-F1 5'-CGAGCCTGGGTAAGGTTCTTC-3'and Rhab16S-R1 5'-CTATCAAAGTGGGGGCCCTTG-3' were designed which amplify a part of the 16S rRNA gene. PCR conditions were as follows: initial denaturation at 95 ˚C for 2 min, followed by 35 cycles of denaturation at 94 ˚C for 30 s, annealing (52 ˚C for *Arsenophonus* and *Spiroplasma*; 54 ˚C for *Wolbachia*, *Rickettsia*, *Cardinium*, *Rhabdochlamydia* and *Orientia*; 55 ˚C for *Serratia*, 30 s), extension (72 ˚C, 90 s) and a final extension at 72 ˚C during 5 min. Electrophoresis was performed on a 1,5 % agarose gel. Gels were stained in a solution of GELRED for approximately 15 min and bands were visualized by UV-fluorescence. The PCR products were sequenced using BigDye v.1.1 Terminator Sequencing mix and run on an ABI 3710 automated sequencer. BLAST searches were used to check primer specificity and to identify the best match of the obtained endosymbiont sequences in the nucleotide collection database. To investigate the presence of multiple strains, sequences were aligned using the Muscle algorithm (Edgar, 2004) implemented in MEGA 5 (Tamura *et al*., 2011).

2.2.2 16S rRNA UNIVERSAL PRIMER CLONING

DNA isolates of three females from the wol- matriline, two from the wol+ matriline and one additional female were used in the cloning assay. As positive PCR bands were present for *Cardinium* and *Rickettsia* in all samples, this ensured that the DNA isolates were of good quality. 16S rRNA sequences were amplified using the universal primers F45 and R1242 (Oneill *et al.*, 1992). PCR conditions were as follows: initial denaturation at 95 ˚C for 2 min, followed by 35 cycles of denaturation at 94 ˚C for 30 s, annealing at 55 ˚C for 30 s, extension (72 ˚C, 90 s) and a final extension at 72 ˚C during 5 min. The PCR amplicons were then ligated into pCR®II-TOPO® vector (Invitrogen) and transferred into TOP10TM chemically competent cells (Invitrogen) according to the manufacturers recommended protocol. Clone sequences were reamplified using

the M13F and M13R primers and presence of the insert was checked by gel electrophoresis. Inserts with bands of expected size were sequenced as described above and a BLAST search was performed to identify the best match in the nucleotide collection database.

2.2.3 DENATURING GEL GRADIENT ELECTROPHORESIS (DGGE)

Females of the Wol-, Wol+ matriline and an additional set of females originating from the Damvallei and Walenbos population were included in the DGGE assay. 16S rRNA sequences were obtained using the previously mentioned universal primers F45 and R1242 (Oneill *et al*., 1992). Next, a nested PCR was performed with use of the primers F357 (5′-CCTACGGGAGGCAGCAG-3′) and R518 (5′-ATTACCGCGGCTGCTGG-3′) which amplify the highly variable V3 region of the 16S rRNA gene (Temmerman *et al*., 2003). A GC-clamp was added to the forward primer to ensure clear DGGE separation (Myers *et al*., 1985, Sheffield *et al*., 1989). PCR conditions were as follows: initial denaturation at 95 ˚C for 1 min, followed by 30 cycles of denaturation at 95 ˚C for 30 s, annealing (55 ˚C for 45 s), extension (72 ˚C, for 60 s) and a final extension at 72 ˚C during 7 min. DGGE analysis was carried out on PCR products using a DCode Universal Mutation Detection System device (Bio-Rad) (as described in Temmerman *et al*., 2003, Hollants *et al*., 2010). Electrophoresis was performed using 35%-70% denaturating gradient polyacrylamide gels at 70 V in 1x TAE buffer at 60 ˚C for 16.5 hours. This allows discrimination of the different amplified 16S rRNA sequences based on nucleotide composition. Gels were placed in the staining solution SYBR gold (Molecular Probes, Invitrogen) for 30 min after which the gel was visualized and photographed with the Molecular Imager Gel Doc XR System. Selected bands were excised by inserting a pipette tip (1 µl) into the gel and incubated overnight at 4°C in TE buffer. A PCR with the primers F357 and R518 (Oneill *et al*., 1992) was performed on the DNA solution and PCR amplicons were sequenced as described above. A BLAST search was performed to obtain the best match in the nucleotide collection database.

2.2.4 ANTIBIOTIC/HEAT TREATMENT

To investigate the bacterial basis of the sex ratio bias in the Wol- matriline, we conducted a heat and antibiotic treatment. 24 offspring of one female (lab generation 4) from the Wol- matriline were randomly assigned to four treatments: (i) distilled water treatment (control), (ii) tetracycline, being a broad spectrum antibiotic (iii) penicillin, which is known to target *Cardinium* but not *Rickettsia* and *Wolbachia* and (iv) a heat treatment in which the offspring were reared at 30°C for seven days after which the temperature was returned to 20°C. Studies show that heat shock treatment targets several endosymbiont species by elimination of the endosymbiont or decreasing bacterial density levels (Van Opijnen and Breeuwer, 1999, Hurst *et al*., 2000, Kyei-Poku *et al*., 2003, Sakamoto *et al*., 2008). Antibiotics were provided by permanently moistening the vials with the antibiotics solution. It has previously been shown that application of antibiotics in this way is effective in targeting endosymbionts in *Oedothorax gibbosus* (Vanthournout *et al*., 2011). After reaching adulthood, females were mated with unrelated males and offspring sex ratio and survival was determined. Female offspring were further mated to increase sample size and offspring sex ratio and survival was determined

(total number of females: n = 8, n = 5, n= 7 and n = 5 for the control, penicillin, tetracycline and heat treatment respectively). Effect of treatment on average clutch sex ratio and juvenile survival was analyzed by means of a generalized linear mixed model (Proc GLIMMIX in SAS v. 9.1.2). Dependence in sex ratio among females was taken into account by including DAM as a random effect. No effect of treatment could be found on juvenile survival ($F_{3, 18.08} = 1.02$; $P = 0.40$) which was generally high for control (mean ± SE: 0.90 ± 0.04), penicillin (mean ± SE: 0.83 ± 0.08), tetracycline (mean ± SE: 0.95 ± 0.03) and heat treated offspring (mean ± SE: 0.94 ± 0.03) indicating that administering antibiotics or exposure to heat does not influence juvenile survival.

3. RESULTS

3.1 WOLBACHIA

A total of 48 females was screened for the Damvallei population of which 20 were found to be uninfected with *Wolbachia*, producing an average sex ratio of 0.5 (mean ± SE: 0.49 ± 0.02; t_{28} = -0.01; P = 0.99). However, one of these females produced only females (table 1). Of the 52 females screened for the Walenbos population, 27 were uninfected with *Wolbachia*. These females equally produced an average sex ratio of 0.5 (mean ± SE: 0.43 ± 0.04; $t_{25.18}$ = -1.65; P = 0.11), of which tree females produced a significantly female biased sex ratio (table 1).

Both the Wol- matriline (mean ± SE: 0.05 ± 0.02; $t_{27.69}$ = -7.65, P < 0.0001) originating from one of these females and the Wol+ matriline (mean ± SE: 0.25 ± 0.04; $t_{23.49}$ = -4.18, P < 0.0001) produced a significantly distorted sex ratio. As the juvenile survival is sufficiently high (≈ 95%), this indicates that the sex ratio bias is not caused by differential sex-specific juvenile mortality. Moreover, females from the Wol- matriline produced significantly more female biased sex ratios than females from the Wol+ matriline ($F_{1, 41.13}$ = 17.49; P < 0.0001). This sex ratio distortion was exclusively maternally inherited as females mated with males originating from this matriline produced equal amounts of male and female offspring (sex ratio: 0.43; P = 0.12). The penetrance of the sex ratio effect is considered to be high as almost all females produced highly distorted sex ratios, contrastingly, in the Wol+ matriline much more variation in the produced sex ratio is observed (figure 1). A positive correlation was found between the sex ratio of a clutch and number of offspring for this Wol- matriline, with significantly fewer males in smaller clutches (weighted Pearson correlation; r = 0.61, P <0.0001, figure 2).

Female	Number of adult males	Total number of offspring	Sex ratio	*P*-value
D-01	0	19	0	< 0.0001
W-01	0	9	0	0.004
W-02	3	15	0.2	0.04
W-03	0	40	0	< 0.0001

Table 1. Overview of the female biased sex ratios produced by *Wolbachia* uninfected females originating from the Damvallei (D) and Walenbos (W) population. P-values represent the probability of difference from a sex ratio of 0.5.

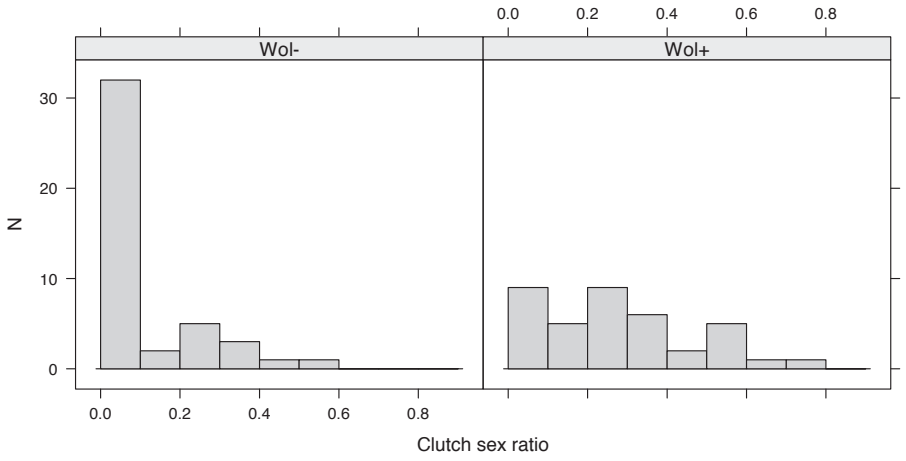


Figure 1. Frequency distribution of the sex ratios produced by females from the Wol- and Wol+ matriline.

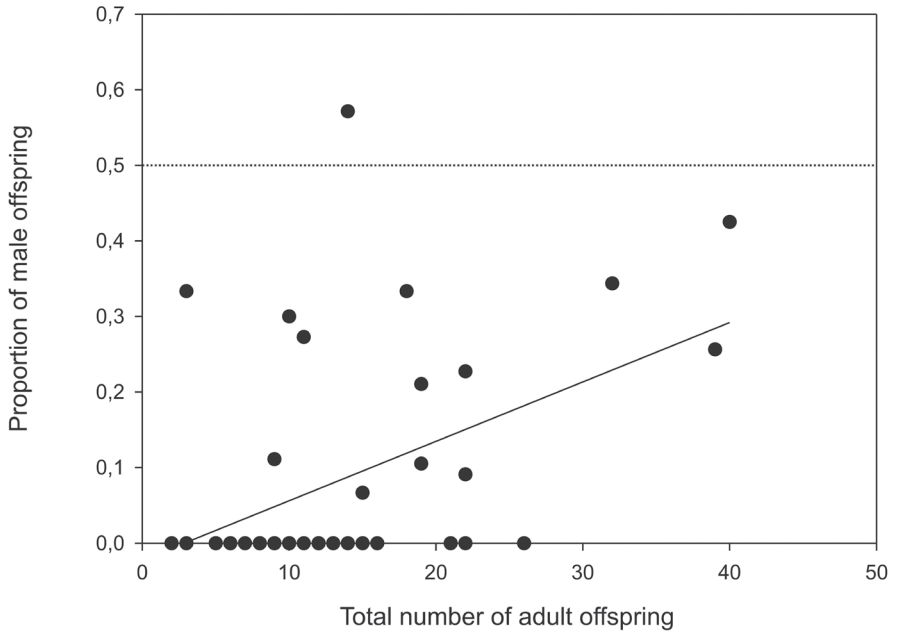


Figure 2. Relationship between proportion of adult offspring and offspring number for females originating from the Wol- matriline. Solid line depicts the linear correlation. The striped line depicts an even sex ratio and is given for illustrative purposes.

3.2 ENDOSYMBIONT SCREENING

Females of the Wol- and Wol+ matriline (n = 10 for each matriline), originating from different generations of lab rearing were screened using species specific primers. All females tested positive for *Cardinium* and *Rickettsia* infection, ensuring that the extracted DNA was of good quality. Clear bands were obtained for all females of the Wol+ matriline when tested for *Wolbachia*. No bands could be detected for females of the Wol- matriline, indicating the absence of *Wolbachia* in this matriline. A subset of these females (n = 3 for each matriline) were screened for *Arsenophonus*, *Spiroplasma*, *Serratia symbiotica* and *Orientia*, which resulted in the absence of clear bands for all endosymbionts except *Arsenophonus*. Bands of varying intensity could be detected for the *Arsenophonus* endosymbiont for all investigated females. However, sequencing and corresponding blast searches revealed that these were amplifications of *Rickettsia* and therefore constituted false positives.

Comparison of the 16S DNA sequences of *Rickettsia* and *Cardinium* did not yield any differences between both matrilines, suggesting that both are infected with the same strains. Hence, based on the specific PCR essays, all females of the Wol- and Wol+ matriline were infected with same strains of *Rickettsia* and *Cardinium* and, with the exception of *Wolbachia*, did not possess a specific endosymbiont community.

As the presence of *Arsenophonus*, *Orientia* and *Serratia symbiotica* was not previously investigated (Vanthournout et al., 2011) field captured females were additionally screened for the presence of *Arsenophonus* (n = 26 for Walenbos and n = 19 for Damvallei), *Orientia* (n = 3 for Walenbos and n = 4 for Damvallei) and *Serratia symbiotica* (n = 3 for Walenbos and n = 1 for Damvallei). No additional positives were found for *Arsenophonus*, indicating that this endosymbiont is not present in the sampled populations. The *Orientia* and *Serratia* symbiotica screening equally did not reveal additional positives suggesting the absence of these endosymbionts, however, as a small number of females was used, a more extensive screening should be performed to obtain more detailed information on the infection frequency of *Orientia* and *Serratia symbiotica*.

3.3 16S rRNA UNIVERSAL PRIMER CLONING

A total of 82 clones were isolated from three different females of the Wol- matriline. No variation was detected in sequences of different females. Sequences of the largest group of 77 clones matched closely with *Rickettsia limonae* (table 2). The five remaining clones were closely related to *Rhabdochlamydia porcellionis* (table 2). The 19 clones of the Wol+ matriline could equally be grouped in two clusters, each containing identical sequences. 13 clones matched with an uncultured alpha proteobacterium (table 2) which is most likely *Wolbachia* as a high similarity was also detected with a *Wolbachia* sp. ([Genbank: CP001391], E-value: <e-199, maximum identity: 98%). Six clones corresponded to *Rickettsia limonae* (table 2). 50 readable clones were obtained for the W-121 female, dividable in three groups. One group comprised three clones containing a sequence that was closely related to *Serratia marcescens* and a group of 46 clones corresponded to *Rickettsia limonae* (table 2). Again, one clone was found to share a high level of relatedness with *Rhabdochlamydia porcellionis* (table 2).

As *Rhabdochlamydia* was found in the distorted Wol- females, and, hence, constitutes a possible candidate causing the sex ratio bias, we designed specific primers for this endosymbiont to screen multiple individuals. This however revealed that *Rhabdochlamydia* infection was not restricted to the mothers that produced a distorted sex ratio, but that all individuals of the Walenbos population are infected (n = 20). Moreover, infection with *Rhabdochlamydia* was not detected in the Damvallei population (n = 18). Primer specificity was subsequently confirmed by sequencing the obtained amplicons and yielded no interspecific variation.

Female	Number of clones	NCBI Nucleotide collection closest match (accession number)	E-value (maximum identity)	NCBI 16S r RNA sequence library closest match (accession number)	E-value (maximum identity)
Wol-	77	*Rickettsia limonae* (AF322443)	<e-199 (99%)	*Rickettsia belii* (NR036774)	<e-199 (95%)
	5	*Rhabdochlamydia porcellionis* (AY223862)	<e-199 (91%)	*Parachlamydia acanthamoebae* (NR026357)	<e-199 (84%)
Wol+	6	*Rickettsia limonae* (AF322443)	<e-199 (95%)	*Rickettsia belii* (NR036774)	<e-199 (91%)
	13	*Uncultered alpha-proteobacterium* (HM111618)	<e-199 (98%)	*Ehrlichia ruminatium* (NR044831)	<e-199 (89%)
W-121	3	*Serratia marcescens* (EU233275)	<e-199 (99%)	*Serratia nematodiphila* (NR044385)	<e-199 (99%)
	1	*Rhabdochlamydia porcellionis* (AY223862)	<e-199 (94%)	*Parachlamydia acanthamoebae* (NR026357)	<e-199 (83%)
	46	*Rickettsia limonae* (AF322443)	<e-199 (96%)	*Rickettsia belii* (NR036774)	<e-199 (93%)

Table 2. Taxonomic affiliation of the clones originating from Walenbos females (Wol- and Wol+ matriline and female W-121).

3.4 DENATURING GEL GRADIENT ELECTROPHORESIS (DGGE)

Comparison of the DGGE pattern between individuals of the Wol+ and Wol- matriline only showed strong discriminating bands that were shared among individuals of the Wol+ matriline (Bands C,D,E,L ; figure 3), but no unique bands that were shared among all offspring of the distorted Wol- matriline. A total of 12 bands (A – L) were selected for sequencing (figure 3; table 3) and revealed that these correspond to identical sequences of *Wolbachia* (C,D,E, and L; *Wolbachia* endosymbiont of *Bemisia tabaci*, table 3), *Rickettsia* (A and B; *Rickettsia* endosymbiont of *Macrolophus* sp., table 3), *Serratia marcescens* (F and G, *Serratia marcescens*, table 3) and *Acinetobacter* (J, *Acinetobacter* sp., table 3). Sequencing of bands H, I and K did not give readable results. Several DNA amplicons from the cloning study were included in the DGGE as a reference. This indeed resulted in one clear band in the DGGE profiles corresponding to *Rickettsia* and *Wolbachia* of the Wol- and Wol+ matrilines respectively.

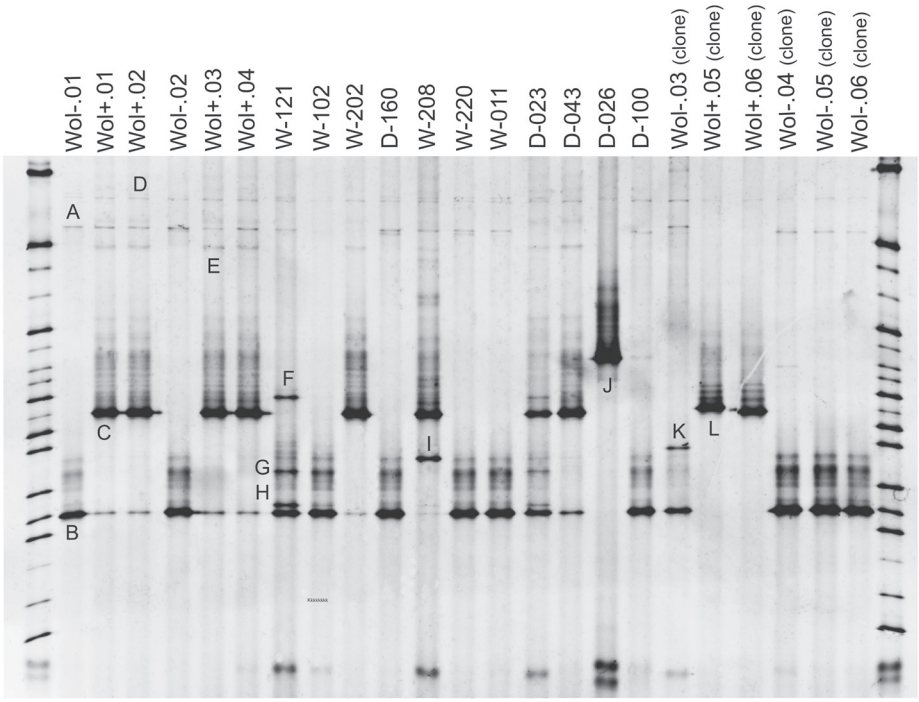


Figure 3. DGGE profiles for the 16S rRNA amplicons of females originating from the Wol- and Wol+ matriline and females of the Damvallei (D) and Walenbos (W) population. Clones indicate the use of DNA amplicons resulting from the cloning study. Bands A and B: *Rickettsia* endosymbiont; C,D,E,L: *Wolbachia* endosymbiont; F and G: *Serratia marcescens*; J: *Acinetobacter* sp.; bands H, I and K did not give readable results.

Female	Bands	NCBI Nucleotide collection closest match (accession number)	*E-value (maximum identity)*
Wol+	C,D,E,L	*Wolbachia* (JQ305707)	<3e-61 (98%)
Wol-	A,B	*Rickettsia* (HE583203)	<e-49 (100%)
W-121	F,G	*Serratia marcescens* (FM163466)	<7e-39 (90%)
D-026	J	*Acinetobacter* (JN228282)	<9e-98 (100%)

Table 3. Taxonomic affilation of the sequenced bands obtained by DGGE of Walenbos females (Wol- and Wol+ matriline, female W-121 and D-026).

3.5 ANTIBIOTICS TREATMENT

Antibiotic and heat treatment of females from the Wol- matriline had a significant effect on the sex ratio produced by a female ($F_{3, 15.81} = 6.94$; $P = 0.003$, figure 4). Control females (mean sex ratio ± SE: 0.09 ± 0.04; $t_{20.25} = -5.25$; $P < 0.0001$) and penicillin treated females (mean sex ratio ± SE: 0.17 ± 0.07; $t_{22} = -3.27$; $P = 0.004$) produced a significantly distorted sex ratio, while heat and tetracycline treated females produced sex ratios not significantly different from 0.5 (mean sex ratio ± SE: 0.49 ± 0.1; $t_{11.97} = -0.20$; $P = 0.84$; mean ± SE: 0.54 ± 0.12; $t12.55 = 0.33$; $P = 0.74$ for heat and tetracycline respectively; figure 4).

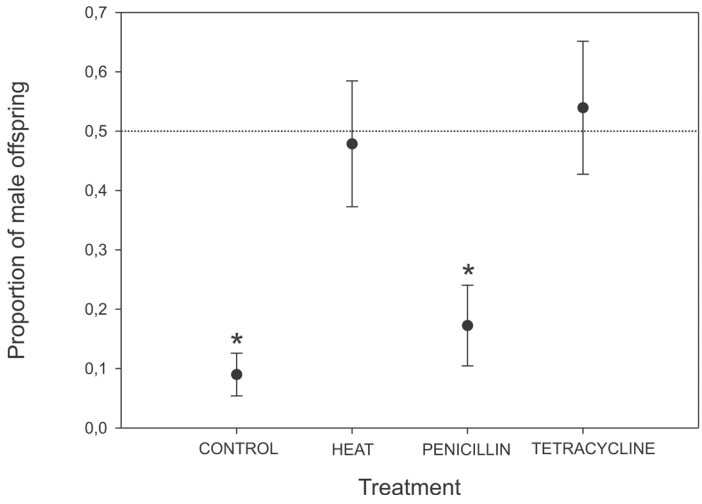


Figure 4. Effect of antibiotics and heat treatment applied to the mother on the average proportion of male offspring. The dotted line indicates a sex ratio of 0.5. Significantly distorted proportions are indicated with an asterisk.

4. DISCUSSION

Previous research on *Oedothorax gibbosus* revealed the presence of a male-killing *Wolbachia* endosymbiont. Results of this study clearly suggest the presence of an additional male-killing cytoplasmic factor as four females produced highly distorted sex ratios in the absence of *Wolbachia*. Moreover, daughters from one of these females persistently produced female biased sex ratios during six generations of lab rearing (Wol- matriline). This bias was clearly maternally inherited as it was only manifested in female offspring. Indeed, when females were mated with males, originating from this matriline, a sex ratio not significantly different from 0.5 was produced, indicating that the sex ratio distortion is not inherited through males. Antibiotic and heat treatment reversed the sex ratio to equal amounts of male and female offspring, demonstrating that the sex ratio distortion has a bacterial basis. As significantly fewer males are present in smaller clutches, the most likely endosymbiont effect is the occurrence of male-killing. However, although these combined results strongly suggest the presence of a male-killing endosymbiont, it remains currently impossible to conclusively assign a particular bacterial strain as a causative agent to the sex ratio distortion.

Endosymbiont specific PCR assays showed that the Wol- matriline is infected with *Rickettsia* and *Cardinium* in females of different generations of lab rearing, but not with *Wolbachia*, *Arsenophonus*, *Orientia*, *Spiroplasma* and *Serratia symbiotica*. Cloning of 16S rRNA amplicons and DGGE confirmed the presence of *Rickettsia* in the Wol- matriline and of *Rickettsia* and *Wolbachia* in the Wol+ matriline. The cloning study additionally revealed the presence of partial 16S sequences that were highly similar to *Rhabdochlamydia porcellionis* in the Wol- matriline and in female W-121. A screening with *Rhabdochlamydia* specific primers revealed that this infection is limited to the Walenbos population as all females in the Walenbos showed infection and none of the Damvallei population.

Chlamydia-like microorganisms have been found in the spider *Coras luctuosus* (Osaki, 1973), however, *Rhabdochlamydia* has not been reported from spiders before and only recently found to constitute a obligatorily intracellular infection in arthropods. *Rhabdochlamydia crassificans* (Corsaro *et al*., 2007) infects cockroaches and causes abdominal swelling of the host. The effects of *Rhabdochlamydia porcellionis* (Kostanjsek *et al*., 2004) in the terrestrial isopod *Porcellio scaber* are currently not known. No effects on the reproductive biology of the host are documented making it unlikely that this endosymbiont species causes the observed sex ratio distortion.

Although *Rickettsia*, *Cardinium* and *Rhabdochlamydia* appear to be fixed in the Walenbos population and no correlation exists between their presence and the occurrence of sex ratio distortion, it cannot be excluded that subtle variations in particular regions of the genome may have profound effects on the phenotypic effects they impose on their host. Significant genomic diversity has been detected in closely related endosymbiont strains, which is the result of high levels of lateral gene transfer, observed in some endosymbiont species (Baldo *et al*., 2006, Moran *et al*., 2008, Ishmael *et al*., 2009, Klasson *et al*., 2009). The production of female biased sex ratios after treatment with penicillin, which targets *Cardinium* but not *Rickettsia*, suggests that *Cardinium* has no direct effect on the sex ratio distortion. The results of the tetracycline and heat treatment indicate that the unknown endosymbiont is susceptible to both treatments.

This points into the direction of *Rickettsia* as this endosymbiont species shows the same susceptibility pattern.

Moreover, individual effects of the different endosymbionts present could be investigated using a q-PCR which quantifies bacterial densities of *Rickettsia*, *Cardinium* and *Rhabdochlamydia* and relating this with the occurrence of the sex ratio effect (Goto *et al*., 2006). Several studies report on natural variation in infection density and interaction effects such as competition between endosymbionts strongly influencing their abundance and hence the penetrance of the reproductive effect on the host (Hurst *et al*., 2000, Goto *et al*., 2006, Unckless *et al*., 2009). Alternatively, the use of low doses of antibiotics and transfection of endosymbionts through microinjection into uninfected hosts could produce singly infected females which can provide more convincing evidence on the individual effects of each endosymbiont (Sasaki *et al*., 2002, Sasaki *et al*., 2005).

Both the cloning study and the DGGE approach demonstrated the presence of *Serratia marcescens* in female W-121. *Serratia marcescens* is associated with arthropods as a potential pathogen (Flyg *et al*., 1980, Lauzon et al., 2003) and is often found in the digestive tract (Lundgren *et al*., 2007) where it is thought to also play a role in the digestive system by producing enzymes or as a regulator of the bacterial community (Tews *et al*., 1996, Adams and Boopathy, 2005, Ruiz-Sanchez *et al*., 2005). As *Serratia marcescens* is commonly found in water and soil (Hejazi and Falkiner, 1997) and the sequence matched closely with the sequence of *Serratia marcescens* found in acid mine rocks [Genbank: FN293172] and on human skin [Genbank: JF127037] contamination of the sample or the presence of the bacteria on the surface of the spider cannot be excluded. Moreover, it has only been detected in a sample originating from a female with an even offspring sex ratio and the screening for *Serratia symbiotica* with species specific primers revealed that this endosymbiont is absent, suggesting that this bacterial species is unlikely to induce sex ratio distortion in *O. gibbosus*. The DGGE analysis revealed an additional presence of *Acinetobacter* in a sample originating from a female of the Damvallei population. This bacterial genus has been found to infect ticks, fleas and mosquitoes and one species of Hemiptera (Murrell *et al*., 2003, Montllor Curley *et al*., 2007, Zouache *et al*., 2009). In *Aedes albopictus*, *Acinetobacter* is found in the salivary glands and hindgut, but not in the reproductive organs, suggesting no effects on host reproduction. Similar to *Serratia*, it is also commonly found in environmental samples (Towner *et al*., 1991), therefore contamination of the sample and presence of the bacterium on the surface of the spider can equally not be ruled out. As it was also found in a sample from a female that produced equal amounts of male and female offspring, this indicates that *Acinetobacter* probably does not influence the sex ratio in this spider species.

Our inability to identify the endosymbiont distorter could also be attributed to the fact that the frequency of the endosymbiont is very low within the infected individuals. Indeed, if the concentration of the bacteria differs several orders of magnitude, isolation of clones and DGGE can lack resolution to detect rare bacterial species. This was for example shown for *Cardinium*. Although this species is present within *O. gibbosus* as shown with primer specific PCR's and subsequent sequencing analysis, it was not picked up with both DGGE and isolation of clones. Therefore, a next generation sequencing approach would be highly recommended to give a more complete picture of the bacterial community present in *O. gibbosus* (Andreotti *et al*., 2011).

Scoring of the different bacterial species, correlating their presence with the occurrence of the sex ratio distortion and performing an antibiotics and heat treatment could yield valuable information on the causative agent of sex ratio distortion in the absence of *Wolbachia*.

Although the identity of the sex ratio distorter cannot be assigned using the molecular techniques in the current study, they independently confirm that *Wolbachia* is absent in this matriline and therefore that an additional sex ratio distorter is present. Moreover, the presence of this additional endosymbiont resulted in the production of highly female biased sex ratios, more distorted compared to *Wolbachia* infected females. The presence of such weaker and stronger male-killers has equally been found in studies of the ladybird *Adalia bipunctata* where four different male-killers have varying effects on the offspring sex ratio (Hurst *et al*., 1999, Majerus *et al*., 2000). However, the two male-killing endosymbionts in *Acraea encedon* both cause the production of all female broods (Jiggins *et al*., 2001). As theoretical models often incorporate weak and strong effects of multiple male-killing endosymbionts in one host species (see below), it would be most interesting to integrate these observed differences of the field situation in the model assumptions.

It is striking that the theoretical prerequisites for stable coexistence of two male-killers are suspected to be all present in *Oedothorax gibbosus*. It has been argued that the evolution of costly suppressor genes acting against a strong male-killer (Randerson *et al*., 2000) allows for a stable male-killer polymorphism. Costly selection against the strong male-killer allows invasion of a weaker male-killing endosymbiont, unaffected by the suppressor gene. As the frequency of the stronger male-killer decreases, so does the frequency of the resistance gene due to the high costs on the host of carrying such a gene. This negative frequency dependent selection creates the necessary conditions for a stable male-killing polymorphism which could be maintained for extended evolutionary periods due to so called Red Queen dynamics, resulting in the occurrence of reciprocal host-endosymbiont interactions that can lead to a long-term co-evolutionary process (Decaestecker *et al*., 2007). The presence of suppressor genes is suspected in *Oedothorax gibbosus* as the inheritance pattern cannot be interpreted as purely maternal and by the occurrence of high numbers of adult males infected with a male-killer. However, performing a specific breeding design is necessary to investigate the presence and precise action of the suppressor genes (Majerus and Majerus, 2010). Interpopulation crosses, performed in chapter 3, indeed suggest the presence of a suppressing nuclear effect in *Oedothorax gibbosus*. Groenenboom & Hogeweg (2002) showed that spatial structure is an important factor in the coexistence of two male-killers. A high spatial subdivision allows for the existence of different niches where the weaker or stronger male-killer is selectively favoured. This spatial pattern formation is also found in *Oedothorax gibbosus* as it occurs exclusively in damp habitats, such as alder carrs, on isolated tufts of moss, surrounded with water. Additionally, a high male mating capacity coupled with low levels of sibmating also produces a stable coexistence of two male-killers (Dannowski *et al*., 2009). Sibmating is expected to occur in *O. gibbosus* due to the strong spatial division of the preferred habitat. Field data concerning the frequency of sibmating is not available for this species. Mating experiments, however, reveal that mating between siblings readily occurs in the lab (pers obs.). These experiments also show high levels of male mating capacity as males can mate with up to four different females, all producing viable offspring (pers. obs.).

The finding of two-male killers in this spider species is congruent with the hypothesis that certain host life history traits play a role in the invasion of male-killers (Hurst, 1991, Hurst and Majerus, 1993, Majerus and Hurst, 1997, Elnagdy *et al*., 2011). Spiders show a high number of similarities with the life history traits of ladybirds known to be infected with multiple species of male-killers. These are egg laying in groups (egg sacs), the occurrence of juvenile cannibalism and high levels of resource competition. In order for a male-killer to invade there must be an advantage for infected females by killing of the males, either directly through the nutritional advantage it holds to consume their dead brothers or indirectly through reduced local resource competition. Both are plausible in spiders. Studies show that spiders are often food limited (reviewed in Wise, 2006), therefore elimination of a potential competitor would result in a higher prey availability. This elimination can also constitute direct nutritional benefits if cannibalism occurs of dead males. High levels of sibling cannibalism were detected in lab studies of *Oedothorax gibbosus* even in the presence of prey items (Vanacker *et al*., 2004). Moreover, cannibalism was more likely to occur between juveniles of different size with larger spiders being more cannibalistic. Therefore, consuming dead brothers before hatching from the egg sac could give infected females an important additional size advantage. These examples show that spiders in general and particularly *Oedothorax gibbosus* show a high number of the life history traits which are thought to facilitate the invasion and spread of male-killing endosymbionts. Moreover, recently it has been additionally found that a male-killing endosymbiont is present in the related species *Oedothorax retusus* (chapter 5). More investigations are clearly needed to determine the frequency of male-killers in the Araneae order and the effects on their host ecology and evolution.

AUTHORS' CONTRIBUTION

Bram Vanthournout and Frederik Hendrickx conceived and designed the study and wrote the manuscript. Bram Vanthournout and Frederik Hendrickx analysed the data and Bram Vanthournout, Frederik Hendrickx and Viki Vandomme performed the breeding experiments and molecular analyses.

ACKNOWLEDGEMENTS

Bram Vanthournout is holder of a PhD scholarship grant from the agency for Innovation by Science and Technology (IWT grant n° 73344). Additional financial support was received from the Belgian Science Policy (BELSPO, research project MO/36/O25).

5. REFERENCES

- ADAMS, L. & BOOPATHY, R. 2005. Isolation and characterization of enteric bacteria from the hindgut of Formosan termite. *Bioresource Technology*, 96, 1592-1598.
- ANDREOTTI, R., DE LEON, A. A. P., DOWD, S. E., GUERRERO, F. D., BENDELE, K. G. & SCOLES, G. A. 2011. Assessment of bacterial diversity in the cattle tick *Rhipicephalus (Boophilus) microplus* through tag-encoded pyrosequencing. *BMC Microbiology*, 11:6.
- BALDO, L., BORDENSTEIN, S., WERNEGREEN, J. J. & WERREN, J. H. 2006. Widespread recombination throughout *Wolbachia* genomes. *Molecular Biology and Evolution*, 23, 437-449.
- BANDI, C., DUNN, A. M., HURST, G. D. D. & RIGAUD, T. 2001. Inherited Microorganisms, Sex-Specific Virulence and Reproductive Parasitism. *Trends in Parasitology*, 17, 88-94.
- BRAIG, H. R., ZHOU, W. G., DOBSON, S. L. & O'NEILL, S. L. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *Journal of Bacteriology*, 180, 2373-2378.
- CHARLAT, S., ENGELSTADTER, J., DYSON, E. A., HORNETT, E. A., DUPLOUY, A., TORTOSA, P., DAVIES, N., RODERICK, G. K., WEDEL, N. & HURST, G. D. D. 2006. Competing Selfish Genetic Elements in the Butterfly *Hypolimnas Bolina*. *Current Biology*, 16, 2453-2458.
- CHARLAT, S., HURST, G. D. D. & MERCOT, H. 2003. Evolutionary Consequences of *Wolbachia* Infections. *Trends in Genetics*, 19, 217-223.
- CORDAUX, R., BOUCHON, D. & GREVE, P. 2011. The impact of endosymbionts on the evolution of host sex-determination mechanisms. *Trends in Genetics*, 27, 332-341.
- CORSARO, D., THOMAS, V., GOY, G., VENDITTI, D., RADEK, R. & GREUB, G. 2007. 'Candidatus *Rhabdochlamydia crassificans*', an intracellular bacterial pathogen of the cockroach *Blatta orientalis* (Insecta : Blattodea). *Systematic and Applied Microbiology*, 30, 221-228.
- DANNOWSKI, J., FLOR, M., TELSCHOW, A. & HAMMERSTEIN, P. 2009. THE EFFECT OF SIBMATING ON THE INFECTION DYNAMICS OF MALE-KILLING BACTERIA. *Evolution*, 63, 2525-2534.
- DAVIS, M. J., YING, Z. T., BRUNNER, B. R., PANTOJA, A. & FERWERDA, F. H. 1998. *Rickettsial* relative associated with Papaya bunchy top disease. *Current Microbiology*, 36, 80-84.
- DECAESTECKER, E., GABA, S., RAEYMAEKERS, J., STOKS, R., VAN KERCKHOVEN, L., EBERT, D. & DE MEESTER, L. 2007. Host-parasite 'Red Queen' dynamics archived in pond sediment. *Nature*, 450 (7171), 870-874.
- DURON, O., BOUCHON, D., BOUTIN, S., BELLAMY, L., ZHOU, L. Q., ENGELSTADTER, J. & HURST, G. D. 2008. The Diversity of Reproductive Parasites Among Arthropods: *Wolbachia* Do Not Walk Alone. *BMC Biology*, 6:27.
- EDGAR, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32, 1792-1797.
- ELNAGDY, S., MAJERUS, M. E. N. & HANDLEY, L. J. L. 2011. The value of an egg: resource reallocation in ladybirds (Coleoptera: Coccinellidae) infected with male-killing bacteria. *Journal of Evolutionary Biology*, 24, 2164-2172.
- ENGELSTADTER, J. & HURST, G. D. D. 2009. The Ecology and Evolution of Microbes That Manipulate Host Reproduction. *Annual Review of Ecology Evolution and Systematics*, 40, 127-149.

- FLYG, C., KENNE, K. & BOMAN, H. G. 1980. INSECT PATHOGENIC PROPERTIES OF *SERRATIA-MARCESCENS* - PHAGE-RESISTANT MUTANTS WITH A DECREASED RESISTANCE TO *CECROPIA* IMMUNITY AND A DECREASED VIRULENCE TO *DROSOPHILA*. *Journal of General Microbiology*, 120, 173-181.
- FUKATSU, T., NIKOH, N., KAWAI, R. & KOGA, R. 2000. The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta : Homoptera). *Applied and Environmental Microbiology*, 66, 2748-2758.
- GOTO, S., ANBUTSU, H. & FUKATSU, T. 2006. Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. *Applied and Environmental Microbiology*, 72, 4805-4810.
- GOTOH, T., NODA, H. & ITO, S. 2007. *Cardinium* Symbionts Cause Cytoplasmic Incompatibility in Spider Mites. *Heredity*, 98, 13-20.
- GROENENBOOM, M. A. C. & HOGEWEG, P. 2002. Space and the persistence of male-killing endosymbionts in insect populations. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 269, 2509-2518.
- HEJAZI, A. & FALKINER, F. R. 1997. *Serratia marcescens*. *Journal of Medical Microbiology*, 46, 903-912.
- HOLLANTS, J., LELIAERT, F., DE CLERCK, O. & WILLEMS, A. 2010. How endo- is endo-? Surface sterilization of delicate samples: a *Bryopsis* (Bryopsidales, Chlorophyta) case study. *Symbiosis*, 51, 131-138.
- HURST, G. D. D., JOHNSON, A. P., VON DER SCHULENBURG, J. H. G. & FUYAMA, Y. 2000. Male-killing *Wolbachia* in *Drosophila*: A temperature-sensitive trait with a threshold bacterial density. *Genetics*, 156, 699-709.
- HURST, G. D. D. & MAJERUS, M. E. N. 1993. WHY DO MATERNALLY INHERITED MICROORGANISMS KILL MALES. *Heredity*, 71, 81-95.
- HURST, G. D. D., VON DER SCHULENBURG, J. H. G., MAJERUS, T. M. O., BERTRAND, D., ZAKHAROV, I. A., BAUNGAARD, J., VOLKL, W., STOUTHAMER, R. & MAJERUS, M. E. N. 1999. Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Molecular Biology*, 8, 133-139.
- HURST, G. D. D. & WERREN, J. H. 2001. The Role of Selfish Genetic Elements in Eukaryotic Evolution. *Nature Reviews Genetics*, 2, 597-606.
- HURST, L. D. 1991. THE INCIDENCES AND EVOLUTION OF CYTOPLASMIC MALE KILLERS. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 244, 91-99.
- ISHMAEL, N., HOTOPP, J. C. D., IOANNIDIS, P., BIBER, S., SAKAMOTO, J., SIOZIOS, S., NENE, V., WERREN, J., BOURTZIS, K., BORDENSTEIN, S. R. & TETTELIN, H. 2009. Extensive genomic diversity of closely related *Wolbachia* strains. *Microbiology-Sgm*, 155, 2211-2222.
- JIGGINS, F. M., HURST, G. D. D., JIGGINS, C. D., VON DER SCHULENBURG, J. H. G. & MAJERUS, M. E. N. 2000. The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. *Parasitology*, 120, 439-446.
- JIGGINS, F. M., HURST, G. D. D., SCHULENBURG, J. & MAJERUS, M. E. N. 2001. Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. *Heredity*, 86, 161-166.

- KLASSON, L., WESTBERG, J., SAPOUNTZIS, P., NAESIUND, K., LUTNAES, Y., DARBY, A. C., VENETI, Z., CHEN, L., BRAIG, H. R., GARRETT, R., BOURTZIS, K. & ANDERSSON, S. G. E. 2009. The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 5725-5730.
- KOSTANJSEK, R., STRUS, J., DROBNE, D. & AVGUSTIN, G. 2004. 'Candidatus *Rhabdochlamydia porcellionis*', an intracellular bacterium from the hepatopancreas of the terrestrial isopod *Porcellio scaber* (Crustacea : Isopoda). *International Journal of Systematic and Evolutionary Microbiology*, 54, 543-549.
- KYEI-POKU, G. K., FLOATE, K. D., BENKEL, B. & GOETTEL, M. S. 2003. Elimination of *Wolbachia* from *Urolepis rufipes* (Hymenoptera : Pteromalidae) with heat and antibiotic treatments: Implications for host reproduction. *Biocontrol Science and Technology*, 13, 341-354.
- LAUZON, C. R., BUSSERT, T. G., SJOGREN, R. E. & PROKOPY, R. J. 2003. *Serratia marcescens* as a bacterial pathogen of *Rhagoletis pomonella* flies (Diptera : Tephritidae). *European Journal of Entomology*, 100, 87-92.
- LUNDGREN, J. G., LEHMAN, R. M. & CHEE-SANFORD, J. 2007. Bacterial communities within digestive tracts of ground beetles (Coleoptera : Carabidae). *Annals of the Entomological Society of America*, 100, 275-282.
- MAJERUS, M. E. N. 2006. The impact of male-killing bacteria on the evolution of aphidophagous coccinellids. *European Journal of Entomology*, 103, 1-7.
- MAJERUS, M. E. N., HINRICH, J., SCHULENBURG, G. V. D. & ZAKHAROV, I. A. 2000. Multiple Causes of Male-Killing in a Single Sample of the Two-Spot Ladybird, *Adalia Bipunctata* (Coleoptera : Coccinellidae) From Moscow. *Heredity*, 84, 605-609.
- MAJERUS, M. E. N. & HURST, G. D. D. 1997. Ladybirds as a model system for the study of male-killing symbionts. *Entomophaga*, 42, 13-20.
- MAJERUS, T. M. O. & MAJERUS, M. E. N. 2010. Intergenomic Arms Races: Detection of a Nuclear Rescue Gene of Male-Killing in a Ladybird. *Plos Pathogens*, 6.
- MAJERUS, T. M. O., VON DER SCHULENBURG, J. H. G., MAJERUS, M. E. N. & HURST, G. D. D. 1999. Molecular Identification of a Male-Killing Agent in the Ladybird *Harmonia axyridis* (Pallas) (Coleoptera : Coccinellidae). *Insect Molecular Biology*, 8, 551-555.
- MONTLLOR CURLEY, C., BRODIE, E. L., LECHNER, M. G. & PURCELL, A. H. 2007. Exploration for facultative endosymbionts of glassy-winged sharpshooter (Hemiptera : Cicadellidae). *Annals of the Entomological Society of America*, 100, 345-349.
- MORAN, N. A., MCCUTCHEON, J. P. & NAKABACHI, A. 2008. Genomics and Evolution of Heritable Bacterial Symbionts. *Annual Review of Genetics*, 42, 165-190.
- MURRELL, A., DOBSON, S. J., YANG, X. Y., LACEY, E. & BARKER, S. C. 2003. A survey of bacterial diversity in ticks, lice and fleas from Australia. *Parasitology Research*, 89, 326-334.
- MYERS, R. M., FISCHER, S. G., MANIATIS, T. & LERMAN, L. S. 1985. MODIFICATION OF THE MELTING PROPERTIES OF DUPLEX DNA BY ATTACHMENT OF A GC-RICH DNA-SEQUENCE AS DETERMINED BY DENATURING GRADIENT GEL-ELECTROPHORESIS. *Nucleic Acids Research*, 13, 3111-3129.
- ONEILL, S. L., GIORDANO, R., COLBERT, A. M. E., KARR, T. L. & ROBERTSON, H. M. 1992. 16s Ribosomal-Rna Phylogenetic Analysis of the Bacterial Endosymbionts Associated With Cytoplasmic Incompatibility in Insects. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 2699-2702.

- OSAKI, H. 1973. Electron microscopic observations of *Chlamydia*-like microorganism in hepatopancreas cells of the spider, *Coras luctusosus*. *Acta Arachnol.*, 25, 23-36.
- RANDERSON, J. P., SMITH, N. G. C. & HURST, L. D. 2000. The evolutionary dynamics of male-killers and their hosts. *Heredity*, 84, 152-160.
- ROUSSET, F. & SOLIGNAC, M. 1995. EVOLUTION OF SINGLE AND DOUBLE *WOLBACHIA* SYMBIOSES DURING SPECIATION IN THE *DROSOPHILA-SIMULANS* COMPLEX. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 6389-6393.
- RUIZ-SANCHEZ, A., CRUZ-CAMARILLO, R., SALCEDO-HERNANDEZ, R. & BARBOZA-CORONA, J. E. 2005. Chitinases from *Serratia marcescens* Nima. *Biotechnology Letters*, 27, 649-653.
- SAKAMOTO, H., KAGEYAMA, D., HOSHIZAKI, S. & ISHIKAWA, Y. 2008. Heat treatment of the adzuki bean borer, *Ostrinia scapulalis* infected with *Wolbachia* gives rise to sexually mosaic offspring. *Journal of Insect Science*, 8.
- SASAKI, T., KUBO, T. & ISHIKAWA, H. 2002. Interspecific transfer of *Wolbachia* between two lepidopteran insects expressing cytoplasmic incompatibility: A *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. *Genetics*, 162, 1313-1319.
- SASAKI, T., MASSAKI, N. & KUBO, T. 2005. *Wolbachia* variant that induces two distinct reproductive phenotypes in different hosts. *Heredity*, 95, 389-393.
- SHEFFIELD, V. C., COX, D. R., LERMAN, L. S. & MYERS, R. M. 1989. ATTACHMENT OF A 40-BASE-PAIR G+C-RICH SEQUENCE (GC-CLAMP) TO GENOMIC DNA FRAGMENTS BY THE POLYMERASE CHAIN-REACTION RESULTS IN IMPROVED DETECTION OF SINGLE-BASE CHANGES. *Proceedings of the National Academy of Sciences of the United States of America*, 86, 232-236.
- STOUTHAMER, R., BREEUWER, J. A. J. & HURST, G. D. D. 1999. *Wolbachia pipientis*: Microbial Manipulator of Arthropod Reproduction. *Annual Review of Microbiology*, 53, 71-102.
- TAKAHASHI, M., URAKAMI, H., YOSHIDA, Y., FURUYA, Y., MISUMI, H., HORI, E., KAWAMURA, A. & TANAKA, H. 1997. Occurrence of high ratio of males after introduction of minocycline in a colony of *Leptotrombidium fletcheri* infected with *Orientia tsutsugamushi*. *European Journal of Epidemiology*, 13, 79-86.
- TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, 28, 2731-2739.
- TEMMERMAN, R., SCHEIRLINCK, I., HUYS, G. & SWINGS, J. 2003. Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, 69, 220-226.
- TEWS, I., VINCENTELLI, R. & VORGIAS, C. E. 1996. N-acetylglucosaminidase (chitobiase) from *Serratia marcescens*: Gene sequence, and protein production and purification in *Escherichia coli*. *Gene*, 170, 63-67.
- TOWNER, K. J., BERGOGNE-BÉRÉZIN, E. & FEWSON, C. A. 1991. *Acinetobacter*: portrait of a genus. In: TOWNER, K. J., BERGOGNE-BÉRÉZIN, E. & FEWSON, C. A. (eds.) *The Biology of Acinetobacter.* Fems symposium no.57.
- UNCKLESS, R. L., BOELIO, L. M., HERREN, J. K. & JAENIKE, J. 2009. *Wolbachia* as populations within individual insects: causes and consequences of density variation in natural populations. *Proceedings of the Royal Society B-Biological Sciences*, 276, 2805-2811.

- VAN OPIJNEN, T. & BREEUWER, J. A. J. 1999. High temperatures eliminate *Wolbachia*, a cytoplasmic incompatibility inducing endosymbiont, from the two-spotted spider mite. *Experimental and Applied Acarology*, 23, 871-881.
- VANACKER, D., DEROOSE, K., PARDO, S., BONTE, D. & MAELFAIT, J. P. 2004. Cannibalism and prey sharing among juveniles of the spider *Oedothorax gibbosus* (Blackwall, 1841) (Erigoninae, Linyphiidae, Araneae). *Belgian Journal of Zoology*, 134, 23-28.
- VANTHOURNOUT, B., SWAEGERS, J. & HENDRICKX, F. 2011. Spiders do not escape reproductive manipulations by *Wolbachia*. *BMC Evolutionary Biology*, 11:15.
- WEEKS, A. R., REYNOLDS, K. T., HOFFMANN, A. A. & MANN, H. 2002. *Wolbachia* Dynamics and Host Effects: What Has (and Has Not) Been Demonstrated? Trends in Ecology & Evolution, 17, 257-262.
- WEISBURG, W. G., BARNS, S. M., PELLETIER, D. A. & LANE, D. J. 1991. 16S RIBOSOMAL DNA AMPLIFICATION FOR PHYLOGENETIC STUDY. *Journal of Bacteriology*, 173, 697-703.
- WERREN, J. H. 1997. Biology of *Wolbachia*. *Annual Review of Entomology*, 42, 587-609.
- WERREN, J. H., BALDO, L. & CLARK, M. E. 2008. *Wolbachia*: Master Manipulators of Invertebrate Biology. *Nature Reviews Microbiology*, 6, 741-751.
- WISE, D. H. 2006. Cannibalism,food limitation, intraspecific competition and the regulation of spider populations. *Annual Review of Entomology*.
- ZOUACHE, K., VORONIN, D., TRAN-VAN, V., MOUSSON, L., FAILLOUX, A. B. & MAVINGUI, P. 2009. Persistent *Wolbachia* and Cultivable Bacteria Infection in the Reproductive and Somatic Tissues of the Mosquito Vector *Aedes albopictus*. *Plos One*, 4.

“Vive la résistance!”

Unknown

CHAPTER 3

INTRAGENOMIC CONFLICT CAUSES LOCAL ADAPTATION IN A DWARF SPIDER

BRAM VANTHOURNOUT [1]

FREDERIK HENDRICKX [1,2]

[1] Terrestrial Ecology Unit, Department of Biology, Ghent University, Ledeganckstraat 35, 9000 Ghent, Belgium

[2] Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium

ABSTRACT

A biased sex ratio induced by selfish genetic elements such as endosymbiont bacteria strongly opposes the Mendelian inheritance of the nuclear genes in the rest of the host genome. Therefore, genetic conflict theory predicts strong selection for the evolution of host nuclear factors suppressing the reproductive effects of the endosymbiont bacterium. Previous research revealed that *Oedothorax gibbosus* is infected with a male-killing *Wolbachia* and a putative second male-killing endosymbiont causing the production of a female biased sex ratio. In line with this, pedigree analysis revealed a clear maternal inheritance pattern of the sex ratio. However, significant additional variation was also detected, suggesting the influence of additional factors, such as suppressor genes, contributing to sex ratio variation. To investigate the presence of such host suppressor genes, inter- and intrapopulation crosses were performed using individuals of two populations. These revealed a strong effect of male nuclear background with males from the Walenbos population performing better in restoring an equal sex ratio, indicating the presence of suppressor genes in this population. This suggests that genetic conflict can cause local adaptation within populations and, moreover, as both populations do not differ in sex ratio produced by a female, that this process can be strongly hidden and only revealed by interpopulation crosses.

1. INTRODUCTION

When components of a genetic system exhibit a different inheritance pattern, this sets the stage for the occurrence of genetic conflict (Cosmides and Tooby, 1981, Werren, 1987, Hurst, 1992, Hurst *et al*., 1996, Werren and Beukeboom, 1998). One potential situation is the presence of selfish genetic elements such as certain endosymbiont bacteria, supernumerary chromosomes and genes exhibiting meiotic drive. As these elements are primarily transmitted by one of the two sexes, distorting the sex ratio of their host may favour their transmission to the next generation. Those endosymbiont bacteria, which obligatorily reside in the cytoplasm of host cells, are only transferred to the next generation via the egg cell of the female. Therefore, high selection pressures exist which bias the sex ratio of the host towards the transmitting female sex. This is achieved through the evolution of different reproductive manipulation strategies that increase the proportion of infected females in the population. These are parthenogenesis induction, male-killing and feminization, which all effect changes in the host offspring sex ratio towards females, and the occurrence of cytoplasmic incompatibility, which decreases the reproductive output of uninfected females when mated with an infected male (Werren, 1997, Stouthamer *et al*., 1999, Charlat *et al*., 2003, Werren *et al*., 2008, Engelstadter and Hurst, 2009). From the viewpoint of nuclear genes of the host, the production of biased amounts of female offspring will lower the proportion of males in the population, which on its turn increases male reproductive value compared to females. This is explained by Fisher's sex-allocation theory, who demonstrated that a 50:50 sex ratio is an evolutionary stable strategy given particular assumptions. In a population with uneven numbers of males and females, the underrepresented sex will have a fitness advantage at the time of mating. As a consequence, selection will favor these genotypes expressing the rarer sex, ultimately leading to the evolution of a stable 50:50 sex ratio (Fisher, 1930, West, 2009).

Hence, due to the difference in inheritance mode between nuclear genes and cytoplasmic bacterial genes, theory predicts strong selection for the evolution of a host's nuclear suppressor counteracting the effects of the endosymbiont genes (Cosmides and Tooby, 1981, Werren, 1987, Hurst, 1992, Hurst *et al*., 1996, Werren and Beukeboom, 1998, but see Jaenike and Dyer, 2008). These genes can act to prevent transmission of the endosymbiont or to silence its phenotypic effects. Indeed, several studies showed that interactions between host and endosymbiont genes readily occur in nature. Transferring bacterial strains across species boundaries indeed revealed negative effects of host genotype on the intensity of the reproductive manipulation (Riegler *et al*., 2004, Tinsley and Majerus, 2007, Kageyama *et al*., 2009) and indicates the possibility of host interference on the reproductive alteration. Within individuals of a single species, bacterial density was found to vary between different host genotypes (Kondo *et al*., 2005). More convincingly, Majerus and Majerus (2010) recently demonstrated the presence of a nuclear rescue factor in the ladybird *Cheilomenes sexmaculata*. This factor consists of a single dominant allele that is expressed in both sexes and suppresses the action of the endosymbiont rather than influence its transmission. A dominant autosomal masculinizing gene that resists the effect of sex ratio distorters is thought to be present in the pill bug *Armadillidium vulgare* (Rigaud and Juchault, 1993) and a recessive allele in *Drosophila prosaltans* (Cavalcanti *et al*., 1957). The high number of adult males infected with a known male-killing endosymbiont in several species of ladybirds suggests the occurrence of suppression of the male killing effect (Weinert *et al*., 2007).

Theoretical models show that suppressor genes acting against the endosymbiont effects are strongly selected and therefore expected to rapidly reach high frequencies in a population (Werren, 1987, Hurst, 1992 and references herein) making it challenging to demonstrate their presence. The manifestation of an apparently new endosymbiont phenotype after transfection into a related species is indicative of the presence of such concealed suppressor genes (McGraw *et al*., 2001, Sasaki *et al*., 2002, Jaenike, 2007). Another possible approach to uncover hidden phenotypes is the investigation of offspring phenotypes originating from interpopulation crosses. In the jumping spider *Habronattus pugillis* for example, reduced offspring viability was observed after interpopulation crosses, which could indicate the presence of cytoplasmic incompatibility (Masta and Maddison, 2002) although this may also be attributed to genetic incompatibility of these isolated populations. A more well studied system is the infection of a male-killing *Wolbachia* in the butterfly *Hypolimnas bolina* (Dyson *et al*., 2002). Females from Polynesian populations exhibit a strong male-killing phenotype resulting in the production of nearly all female broods. Females of South East Asian populations in contrast produce even sex ratios with the same *Wolbachia* strain being present in all males (Charlat *et al*., 2005, Hornett *et al*., 2006). Crosses between the populations revealed that this difference in male-killing expression arose due to the presence of a suppressor gene in the South East Asian butterflies.

Previous research on the dwarf spider *Oedothorax gibbosus* revealed the presence of a male-killing *Wolbachia* endosymbiont, which causes a significantly female biased sex ratio of approximately 30% males (Vanthournout *et al*., 2011). Comparison between two populations revealed a similar infection rate and sex ratio bias. Besides the *Wolbachia* infection, a yet unidentified endosymbiont is suspected to cause a highly distorted sex ratio of only 5% males in individuals from the Walenbos population. Moreover, it was found that infection rate of particular endosymbionts may differ strongly between both populations as shown for *Rhabdochlamydia*, where fixation was observed for females of the Walenbos population, while none of the screened Damvallei individuals showed the infection.

Hence, given the fact that populations, which produce similar average sex ratios, may strongly differ in endosymbiont community at a relatively small scale, and that endosymbiont infection does not explain all sex ratio variation in this species (Vanthournout *et al*. 2011), it can be expected that local adaptation to suppress the effect of endosymbiont induced sex ratio distortion is acting. We test this hypothesis by performing inter- and intrapopulation crosses using individuals of the Damvallei and Walenbos.

2. MATERIAL AND METHODS

2.1 INTER- AND INTRAPOPULATION CROSSES

Juvenile male and female spiders were captured by hand in two populations, Damvallei (D) and Walenbos (W), in 2010 and 2011. Spiders were reared individually under standardized conditions (Vanthournout *et al*., 2011) till adulthood. To test for the action of different suppression genes and to determine any potentially hidden phenotypes within these populations of *Oedothorax gibbosus*, intra- and reciprocal interpopulation matings were performed that resulted in four different types of crosses: D x D, D x W, W x D and W x W (♂ x ♀; Table 1). Spiders were mated according to a half-sib mating scheme wherein each male was mated with females from both the Damvallei and Walenbos population. To correct for possible effects of male mating order, half of the Damvallei males were first mated with a Damvallei female and half with a Walenbos female. Subsequent matings were performed with females from the alternate population. A similar strategy was applied for males of the Walenbos population. Males were placed in the vial of the female for at least 24 hours and afterwards stored in ethanol (97%). Females were allowed to lay up to four egg sacs in the vial they were mated in and were subsequently stored in ethanol (97%). Offspring were reared individually under the same standardized conditions and upon reaching adulthood, sex of the spider was determined by visual inspection using a stereomicroscope.

Significance of the effect of male and female population and female *Wolbachia* infection status, as determined by performing specific PCR's (see Vanthournout *et al*. 2011 for details), and all their respective interactions were determined using a generalized linear mixed model (proc GLIMMIX in SAS v. 9.1.2) with a binomial error structure and logit link function. As a half sib breeding design was used, male id was included as a random effect to account for the dependence of sex ratios resulting from females mated with the same male.

Crossing type (♂x♀)	Number of lines	Total number of offspring
D x D	22	694
D x W	23	560
W x D	26	763
W x W	29	671

Table 1. Overview of the number of lines and total number of offspring for the different inter- and intrapopulation crosses.

It was found that females from the D x W (♂ x ♀) cross produce a highly female biased sex ratio (see results 3.1). To verify the effect of nuclear and cytoplasmic population origin, the nuclear background of these F1 hybrid offspring was enriched for both a Damvallei (D introgression)

and Walenbos (W introgression) nuclear background by crossing female offspring with pure males of Damvallei and Walenbos respectively. To investigate the bacterial basis of this distortion, four D x W females were treated with the broad spectrum antibiotic tetracycline hydrochloride which targets bacteria of different families (0.1%, w/v; 0.002M), as described in Vanthournout *et al.* (2011), and subsequently mated with pure Damvallei lines. Effect of male population origin was examined using a generalized linear mixed model (proc GLIMMIX in SAS v. 9.1.2) with male population as a fixed effect. Female matriline was included as a random effect to account for dependence of sex ratios resulting from the same matriline.

2.2 ESTIMATION OF INTRAPOPULATION VARIATION IN RESTORING SEX RATIO DISTORTION

To test if intrapopulation variation is present in the capacity to restore sex ratio distortion caused by two different endosymbionts, field collected Walenbos males were mated with females from two Walenbos matrilines (Wol- and Wol+) using a half-sib breeding design. Females from the matriline Wol- resulted from the 6th generation of lab rearing and are infected with a yet unidentified endosymbiont factor causing a highly distorted sex ratio with the production of almost all-female clutches (chapter 2). Wol+ females also resulted from a 6th generation lab rearing and this matriline shows infection with a male-killing *Wolbachia*, which causes the production of female biased clutches (30% males) (chapter 2). To test for variation in males in their ability to restore the sex ratio, males were incorporated as a random effect in a generalized linear model, and the variance in the sex ratio produced among males was estimated and tested if it is significantly larger than zero. It is important to note that this model only addresses the question if there is significant variation among males to restore the sex ratio's of *both* matrilines simultaneously. To test if male specific effects differs among matrilines, we also estimated a male*matriline random effect and tested its significance. More specifically, an unstructured covariance matrix was implemented that estimates the variation among males to restore the sex ratio *within* each matriline, as well as their correlation *among* matrilines. Models were run with the GLIMMIX procedure in SAS v. 9.1.2.

3.RESULTS

3.1 INTER- INTRAPOPULATION CROSSES

Wolbachia infection status of the female, and male and female population origin significantly affect the clutch sex ratio produced by a female (table 2, figure 1). Remarkably, male and female population origin affected sex ratio in an opposite direction. While W females produced a significantly more female biased sex ratio compared to D females, the effect of male population origin was reversed with D males having a more female biasing effect compared to W males. In accordance with previous results *Wolbachia* infection status of the female lowered the proportion of male offspring significantly across male and female population origins. However, the effect of *Wolbachia* and male and female population origin did not affect sex ratio in a mere additive way. When W females were crossed with D males, the produced sex ratio was even more directed towards female biased clutches as would be expected from the additive female biasing effects of W females and D males only (table 2: male x female population interaction; figure 1). Moreover, this interaction was dependent of *Wolbachia* infection status of the female and much more pronounced when females are infected with *Wolbachia* (table 2: three-way interaction; figure 1).

To further confirm the female biasing effect of D males versus the restoring effect of W males, offspring of the D x W cross, from both *Wolbachia* infected and uninfected females, were mated with both pure D and W males. The sex ratio produced by these D x W offspring was significantly affected by the population origin of their mates in a direction consistent with the previously obtained results ($F_{2, 24} = 8.15$; $P = 0.002$; figure 2). Hence, introgression of the D background into this hybrid nuclear and Walenbos cytoplasmic background (mating of a D x W female with a D X D male) retained the highly distorted sex ratio (mean $\pm$ SE: 0.25 $\pm$ 0.05; $t_{12.65} = -4.36$; $P = 0.0008$), while introgressing a Walenbos background (mating of a D x W female with a W X W male) resulted in the production of an even sex ratio (mean $\pm$ SE: 0.38 $\pm$ 0.06; $t_{12.07} = -1.92$; $P = 0.08$). For this analysis based on a few maternal lines only, no significant effect of *Wolbachia* infection status of the parental females was present ($F_{1, 4.3} = 1.39$, $P = 0.3$). However, to explicitly test if the female bias in clutches produced by D x W females that were mated with pure D x D males is caused by endosymbiont bacteria, a subset was treated with the broad spectrum antibiotic tetracycline. As expected, this treatment significantly restored an equal sex ratio (mean $\pm$ SE: 0.48 $\pm$ 0.08; $t_{20.17} = -0.21$; $P = 0.84$, figure 2).

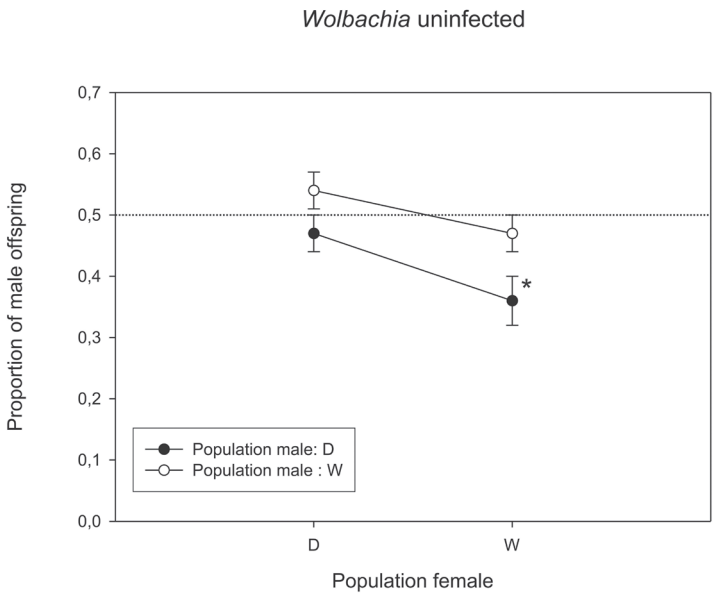
3.2 ESTIMATION OF INTRAPOPULATION VARIATION IN RESTORING SEX RATIO DISTORTION

Significant variation exists between males in their capacity to restore a female biased sex ratio when mated with females from the Wol- (estimate = 1.66, SE = 0.95, $P = 0.04$) and Wol+ matriline (estimate = 0.49, SE = 0.26, $P = 0.03$, figure 3). However, males that were able to restore the sex ratio in one matriline did not necessarily restore sex ratio in the other

matriline as shown by a weak correlation across matrilines that was significantly lower than 1 (r = 0.19, SE = 0.42; P = 0.03). Consistent with previously obtained results (chapter 2), a highly significant effect of matriline was found on the sex ratio produced by females ($F_{1, 30.04}$ = 13.57; P = 0.0009), with females originating from the Wol- matriline producing an even more female biased sex ratio (mean ± SE: 0.07 ± 0.02; significance of female bias: $t_{37.91}$ = -7.96; P <0.0001) compared to females of the *Wolbachia* infected matriline (mean ± SE: 0.25 ± 0.05, $t_{21.86}$ = -4.36; P = 0.0003).

Source	*F*	*P*-value
Fixed effects		
Wolbachia infection status female	27.87	<0.0001
Population male	13.89	0.0008
Population female	30.18	<0.0001
Pop male * Pop female	5.99	0.017
Pop male * Wol female	0.32	0.57
Pop female * Wol female	5.47	0.02
Pop male * Pop female * Wol female	4.89	0.03
Random effect		
Male	0.05	n.s.

Table 2. Effect of male and female population origin and *Wolbachia* infection status and their respective interactions on clutch sex ratio as analyzed by means of a generalized linear model.



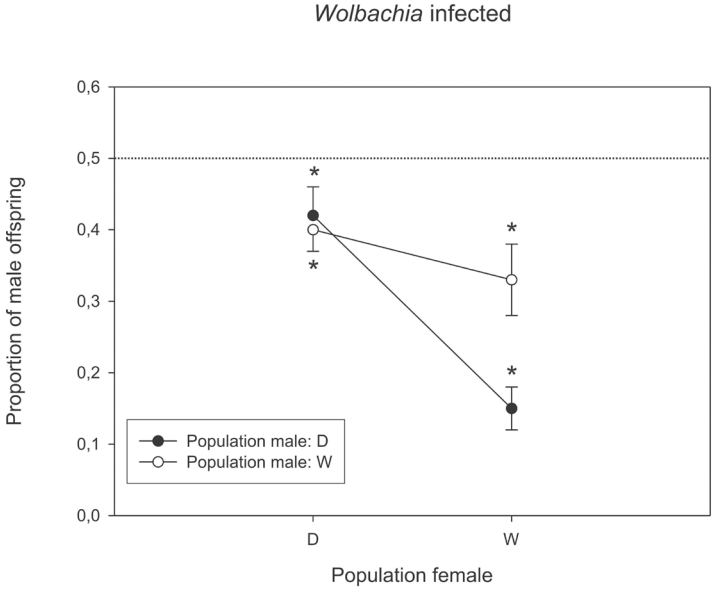


Figure 1. Effect of male and female population origin (D = Damvallei and W = Walenbos) on the average proportion of male offspring produced by *Wolbachia* uninfected and *Wolbachia* infected females. The striped line depicts an even sex ratio. An asterisk indicates sex ratios significantly different from 0.5.

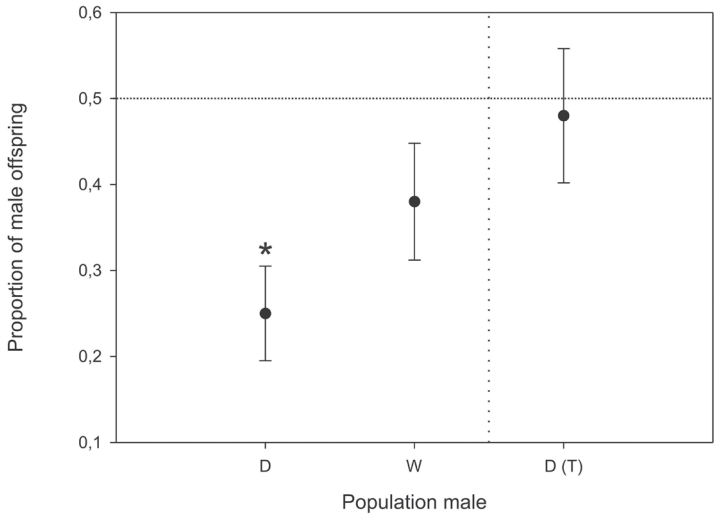


Figure 2. Average proportion of male offspring produced by a female offspring originating from the D x W (♂ x ♀) cross when mated with a Damvallei (D) and Walenbos (W) male. D(T) indicates that a Damvallei male was mated with a tetracycline treated female. The striped line depicts a sex ratio of 0.5. An asterisk indicates sex ratios significantly different from 0.5.

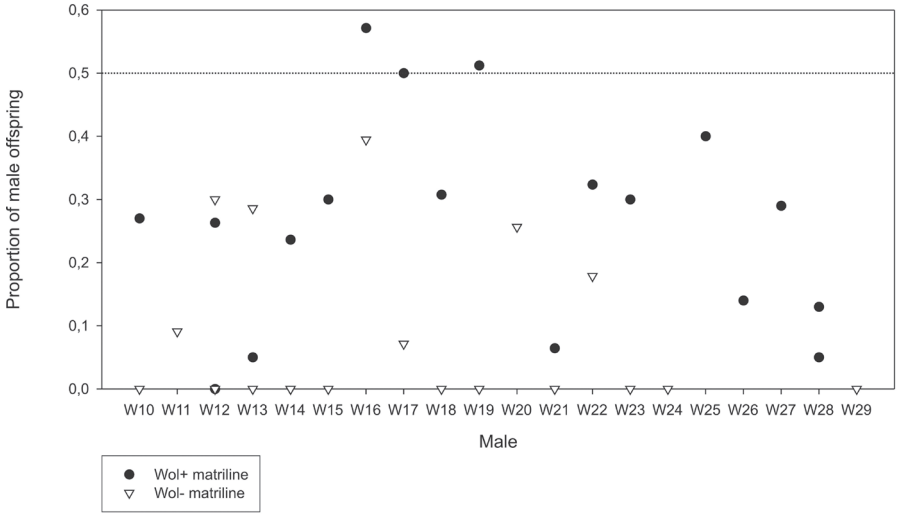


Figure 3. Overview of the sex ratios produced by females of the Wol+ (filled circles) and Wol- matriline (empty triangles) when mated with different field collected males. Sex ratios are given which are based on at least 10 offspring. The striped line depicts a sex ratio of 0.5.

4. DISCUSSION

Infection with maternally inherited endosymbiont bacteria is thought to impose strong selective forces on the host as their uniparental inheritance pattern conflicts with the biparental inheritance pattern of the hosts' autosomal genes (Cosmides and Tooby, 1981, Werren, 1987, Hurst, 1992, Werren and Beukeboom, 1998). Theory therefore predicts the evolution of host factors which suppress the effects of the endosymbiont genes. Our results are in strong concordance with the presence of such genetic conflict by demonstrating a significant effect of nuclear population background on an identical cytoplasmic background.

The results of the inter- and intrapopulation crosses strongly suggest that more effective suppressor genes are present in the Walenbos population to counteract the sex ratio distorting effect of the endosymbiont community in this population. Indeed, females originating from the W population produced a significantly more female biased sex ratio compared to D females, irrespective of the population origin of their mates. Hence, if genetic conflict would act to restore this stronger female bias observed for W females, stronger selection on nuclear genes to restore this bias is expected in this W population. This was confirmed by performing interpopulation crosses as males of the W population appeared more effective compared to D males in producing a more equal sex ratio, irrespective of the female population origin. Remarkably, these counteracting selection effects on the nuclear (host) and the cytoplasmic (endosymbiont) background results in no detectable difference in the sex ratio of pure bred females of both populations. This demonstrates that the ongoing arms race between endosymbionts and their hosts can be strongly hidden and only unmasked when different populations are crossed reciprocally.

The two populations used in the current study have been previously shown to be infected with a sex ratio distorting *Wolbachia* endosymbiont (Vanthournout *et al.* 2011). The effect of a *Wolbachia* infection on the sex ratio is again confirmed as female biased offspring sex ratios are produced by infected females from the four inter and intra-population crosses. Yet, this sex ratio biasing effect of *Wolbachia* is strongly dependent on the interaction with male and female population. This is illustrated by the highly female biased sex ratio produced by *Wolbachia* infected females from the Walenbos when mated with males originating from the Damvallei population (D x W cross, ♂ x ♀). As clutches originating from this cross are more female biased than the intrapopulation cross W x W, male population appears to have a very strong effect on the offspring sex ratio. The effect of male population is indeed supported by introgressing nuclear genes of the two populations in the cytoplasmic background of the Walenbos population. Enriching D male x W female hybrids with nuclear genes from Walenbos resulted in the production of an even sex ratio, demonstrating the restoring capacity of Walenbos males which is indicative for the presence of suppressor genes in this population. As an even sex ratio is obtained in the first generation offspring, this suggests that the suppressor acts zygotically. In contrast, when D male x W female hybrids were introgressed with a Damvallei nuclear background by mating them with pure Damvallei males, the distorted sex ratio of the F1 hybrids was retained. The bacterial basis of the female biased sex ratio distortion was confirmed by the tetracycline treatment of females mated with Damvallei males as this causes the production of an even sex ratio.

Although these data suggest that females of both populations harbor *Wolbachia* strains that differ in their capacity to distort sex ratios, no variation was previously detected in the *wsp* (*Wolbachia* surface protein) sequences from both populations. However, mutations in other parts of the genome could have strong effects on the penetrance of the sex ratio effect. Natural variation in *Wolbachia* density, known to be correlated with the severity of the reproductive effect, as caused by e.g. variation in the frequency of bacteriophages (Bordenstein *et al*., 2006) and interactions with other endosymbiont species could account for the observed differences in sex ratio effect between the *Wolbachia* endosymbionts of both populations.

Interestingly, *Wolbachia* uninfected females of the interpopulation cross D x W also produce a slight though significant female biased sex ratio which could indicate that a similar though less pronounced mechanism is active in absence of *Wolbachia*. Previous endosymbiont species specific PCR revealed that *Arsenophonus*, *Orientia* and *Serratia symbiotica* are most likely absent in the Damvallei and Walenbos population (chapter 2). Both populations show fixation of *Cardinium* and *Rickettsia* endosymbionts, making it at first sight unlikely that one of these species could be the causative agent of the female biased sex ratio distortion in *Wolbachia* uninfected females. However, it cannot be excluded that different strains exist in the two populations. Although sequence analysis revealed no interpopulation variation in *Cardinium* 16S sequences and *Rickettsia citrate* sequences, multiple loci should be investigated to decisively answer this question. The presence of different strains of *Cardinium* or *Rickettsia* has also been hypothesized to account for the effects of the putative endosymbiont in the Walenbos population (Wol- matriline, chapter 2). However, it is unlikely that this particular unidentified endosymbiont is responsible for the sex ratio distortion in interpopulation crosses. Several generations of lab rearing resulted in the production of highly female biased sex ratios even when mated with males of the Walenbos, revealing little suppression of the sex ratio effect.

Moreover, previous molecular screening of both populations revealed that individuals of both populations can be infected with different endosymbionts, as shown for *Rhabdochlamydia* which was found in all investigated female individuals of the Walenbos populations, but not in the individuals captured at Damvallei. This pattern of infection suggests that *Rhabdochlamydia* could cause the sex ratio bias in interpopulation crosses in the absence of *Wolbachia*. *Rhabdochlamydia* has been reported in isopods (Kostanjsek *et al*., 2004) and cockroaches (Corsaro *et al*., 2007), with no effects on the host reproduction currently recorded for both species, making it unlikely that this endosymbiont causes the sex ratio distortion in the absence of *Wolbachia*. However, using antibiotics that target the *Rhabdochlamydia* infection and transferring *Rhabdochlamydia* isolates from Walenbos females into Damvallei females could be a first step to determine the effects of this endosymbiont on host reproduction and clarifying its potential role in interpopulation crosses. As it has been shown that both populations differ in endosymbiont community there is a clear need to investigate this further in order to obtain a clear view on the endosymbiont species richness. A next generation sequencing approach would allow us to obtain a much more exhaustive assessment of the bacterial community and a quantification of the endosymbiont diversity in both the Damvallei and Walenbos population (Andreotti *et al*., 2011).

Selection for genes to suppress a sex ratio bias assumes that variation should exist in the capacity to restore a female biased sex ratio within a population. This was demonstrated in our second experiment mating males of the Walenbos with females of two female biased matrilines. Significant variation exists in the ability of males to restore an even sex ratio, although this effect was strongly dependent on female matriline as females of the Wol- matriline produced a more female biased sex ratio compared to females from the Wol+ matriline. Moreover, the action of the suppressing mechanism seems not to be consistent for both the Wol- and Wol+ endosymbiont as males capable of restoring an even sex ratio in Wol+ females were not able to produce a high number of males when mated with females of the Wol- matriline and, albeit to a lesser extent, vice versa.

Currently, only a small number of host species have been identified to harbour genes that suppress the action of phenotypic effects exerted by endosymbionts. Most extensively studied are ladybirds (Majerus and Majerus, 2010) and butterflies (Hornett *et al.*, 2006). It was shown that in the ladybird *Cheilomenes sexmaculata* a nuclear rescue factor is present that counteracts the effects of a male-killing γ-proteobacterium. In the butterfly *Hypolimna bolinas* marked differences are observed between populations originating from the outer margins of the species range despite being infected with the same male-killing *Wolbachia* endosymbiont (Hornett *et al.*, 2006). Females from Polynesian populations produce a highly distorted sex ratio with the production of almost all female broods while South East Asian females produce even sex ratios. Interpopulation crosses revealed that South East Asian females when mated with Polynesian males produce female biased sex ratios after two generations of introgression. Crossing of Polynesian females with males from South East Asia returns the sex ratio back to 0.5 after one generation, demonstrating the presence of zygotically acting suppressor genes in the South East Asian populations which are absent in the Polynesian populations. This population dependent distribution of suppressor genes is the driving force behind the distinct differences in sex ratio between the Polynesian and South East Asian populations. Contrastingly, in *Oedothorax gibbosus* no difference between the average sex ratio of the Damvallei (0.42 ± 0.03) and Walenbos (0.39 ± 0.02) is detected, showing that endosymbiont effects can be highly masked by the action of suppressor genes. Only by performing interpopulation crosses it was revealed that the penetrance of the reproductive effect of *Wolbachia* is suppressed in the Walenbos population, demonstrating that genetic conflict can cause populations to adapt locally with the evolution of specific suppressor genes rendering the endosymbiont effect quiescent. This lack of phenotypic differentiation between populations could lead to an underestimation of both the frequency of reproductive effects of endosymbionts on their hosts and the rate of evolution of suppressor genes. At least for the Araneae order this could explain the discrepancy between the high number of spider species infected with endosymbionts and the paucity of cases in which their effects have been demonstrated. Moreover, these findings have implications in the field of conservation biology as reconnecting locally adapted populations could have strong, short-term deleterious effects.

AUTHORS' CONTRIBUTION

Bram Vanthournout and Frederik Hendrickx conceived and designed the study and wrote the manuscript. Bram Vanthournout and Frederik Hendrickx analysed the data and performed the breeding experiments.

ACKNOWLEDGEMENTS

Bram Vanthournout is holder of a PhD scholarship grant from the agency for Innovation by Science and Technology (IWT grant n° 73344). Additional financial support was received from the Belgian Science Policy (BELSPO, research project MO/36/O25).

5. REFERENCES

- ANDREOTTI, R., DE LEON, A. A. P., DOWD, S. E., GUERRERO, F. D., BENDELE, K. G. & SCOLES, G. A. 2011. Assessment of bacterial diversity in the cattle tick *Rhipicephalus (Boophilus) microplus* through tag-encoded pyrosequencing. *BMC Microbiology*, 11:6.
- BORDENSTEIN, S. R., MARSHALL, M. L., FRY, A. J., KIM, U. & WERNEGREEN, J. J. 2006. The Tripartite Associations Between Bacteriophage, *Wolbachia*, and Arthropods. Plos Pathogens, 2, 384-393.
- CAVALCANTI, A. G. L., FALCAO, D. N. & CASTRO, L. E. 1957. SEX-RATIO IN *DROSOPHILA-PROSALTANS* - A CHARACTER DUE TO INTERACTION BETWEEN NUCLEAR GENES AND CYTOPLASMIC FACTORS. *American Naturalist*, 91, 327-329.
- CHARLAT, S., HORNETT, E. A., DYSON, E. A., HO, P. P. Y., LOC, N. T., SCHILTHUIZEN, M., DAVIES, N., RODERICK, G. K. & HURST, G. D. D. 2005. Prevalence and penetrance variation of male-killing *Wolbachia* across Indo-Pacific populations of the butterfly *Hypolimnas bolina*. *Molecular Ecology*, 14, 3525-3530.
- CHARLAT, S., HURST, G. D. D. & MERCOT, H. 2003. Evolutionary Consequences of *Wolbachia* Infections. *Trends in Genetics*, 19, 217-223.
- CORSARO, D., THOMAS, V., GOY, G., VENDITTI, D., RADEK, R. & GREUB, G. 2007. 'Candidatus *Rhabdochlamydia crassificans*', an intracellular bacterial pathogen of the cockroach *Blatta orientalis* (Insecta : Blattodea). *Systematic and Applied Microbiology*, 30, 221-228.
- COSMIDES, L. M. & TOOBY, J. 1981. CYTOPLASMIC INHERITANCE AND INTRAGENOMIC CONFLICT. *Journal of Theoretical Biology*, 89, 83-129.
- DYSON, E. A., KAMATH, M. K. & HURST, G. D. D. 2002. *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera : Nymphalidae): evidence for horizontal transmission of a butterfly male killer. Heredity, 88, 166-171.
- ENGELSTADTER, J. & HURST, G. D. D. 2009. The Ecology and Evolution of Microbes That Manipulate Host Reproduction. *Annual Review of Ecology Evolution and Systematics*, 40, 127-149.
- FISHER, R. A. 1930. The genetical theory of natural selection., Oxford University Press.
- HORNETT, E. A., CHARLAT, S., DUPLOUY, A. M. R., DAVIES, N., RODERICK, G. K., WEDELL, N. & HURST, G. D. D. 2006. Evolution of Male-Killer Suppression in a Natural Population. *Plos Biology*, 4, 1643-1648.
- HURST, L. D. 1992. INTRAGENOMIC CONFLICT AS AN EVOLUTIONARY FORCE. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 248, 135-140.
- HURST, L. D., ATLAN, A. & BENGTSSON, B. O. 1996. Genetic conflicts. *Quarterly Review of Biology*, 71, 317-364.
- JAENIKE, J. 2007. Spontaneous emergence of a new *Wolbachia* phenotype. *Evolution*, 61, 2244-2252.
- JAENIKE, J. & DYER, K. A. 2008. No resistance to male-killing *Wolbachia* after thousands of years of infection. *Journal of Evolutionary Biology*, 21, 1570-1577.
- KAGEYAMA, D., ANBUTSU, H., SHIMADA, M. & FUKATSU, T. 2009. Effects of host genotype against the expression of *spiroplasma*-induced male killing in *Drosophila melanogaster*. *Heredity*, 102, 475-482.
- KONDO, N., SHIMADA, M. & FUKATSU, T. 2005. Infection density of *Wolbachia* endosymbiont affected by co-infection and host genotype. *Biology Letters*, 1, 488-491.

- KOSTANJSEK, R., STRUS, J., DROBNE, D. & AVGUSTIN, G. 2004. 'Candidatus *Rhabdochlamydia porcellionis*', an intracellular bacterium from the hepatopancreas of the terrestrial isopod *Porcellio scaber* (Crustacea : Isopoda). *International Journal of Systematic and Evolutionary Microbiology*, 54, 543-549.
- MAJERUS, T. M. O. & MAJERUS, M. E. N. 2010. Intergenomic Arms Races: Detection of a Nuclear Rescue Gene of Male-Killing in a Ladybird. *Plos Pathogens*, 6.
- MASTA, S. E. & MADDISON, W. P. 2002. Sexual Selection Driving Diversification in Jumping Spiders. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 4442-4447.
- MCGRAW, E. A., MERRITT, D. J., DROLLER, J. N. & O'NEILL, S. L. 2001. *Wolbachia*-mediated sperm modification is dependent on the host genotype in *Drosophila*. *Proceedings of the Royal Society of London Series B-Biological Sciences,* 268, 2565-2570.
- RIEGLER, M., CHARLAT, S., STAUFFER, C. & MERCOT, H. 2004. *Wolbachia* transfer from *Rhagoletis cerasi* to *Drosophila simulans*: Investigating the outcomes of host-symbiont coevolution. *Applied and Environmental Microbiology*, 70, 273-279.
- RIGAUD, T. & JUCHAULT, P. 1993. CONFLICT BETWEEN FEMINIZING SEX-RATIO DISTORTERS AND AN AUTOSOMAL MASCULINIZING GENE IN THE TERRESTRIAL ISOPOD *ARMADILLIDIUM-VULGARE* LATR. *Genetics*, 133, 247-252.
- SASAKI, T., KUBO, T. & ISHIKAWA, H. 2002. Interspecific transfer of *Wolbachia* between two lepidopteran insects expressing cytoplasmic incompatibility: A *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. *Genetics*, 162, 1313-1319.
- STOUTHAMER, R., BREEUWER, J. A. J. & HURST, G. D. D. 1999. *Wolbachia pipientis*: Microbial Manipulator of Arthropod Reproduction. *Annual Review of Microbiology*, 53, 71-102.
- TINSLEY, M. C. & MAJERUS, M. E. N. 2007. Small steps or giant leaps for male-killers? Phylogenetic constraints to male-killer host shifts. *BMC Evolutionary Biology*, 7:238.
- VANTHOURNOUT, B., SWAEGERS, J. & HENDRICKX, F. 2011. Spiders do not escape reproductive manipulations by Wolbachia. *BMC Evolutionary Biology*, 11:15.
- WEINERT, L. A., TINSLEY, M. C., TEMPERLEY, M. & JIGGINS, F. M. 2007. Are we underestimating the diversity and incidence of insect bacterial symbionts? A case study in ladybird beetles. *Biology Letters*, 3, 678-681.
- WERREN, J. H. 1987. THE COEVOLUTION OF AUTOSOMAL AND CYTOPLASMIC SEX-RATIO FACTORS. *Journal of Theoretical Biology*, 124, 317-334.
- WERREN, J. H. 1997. Biology of *Wolbachia*. *Annual Review of Entomology*, 42, 587-609.
- WERREN, J. H., BALDO, L. & CLARK, M. E. 2008. *Wolbachia*: Master Manipulators of Invertebrate Biology. *Nature Reviews Microbiology*, 6, 741-751.
- WERREN, J. H. & BEUKEBOOM, L. W. 1998. Sex Determination, Sex Ratios, and Genetic Conflict. *Annual Review of Ecology and Systematics*, 29, 233-261.
- WEST, S. A. 2009. *Sex Allocation*, Princeton University Press.

“Stain them all, let the flow cytometer sort them out!”

modified from “Kill them all, let God sort them out.”
Arnaul Amalric 1209

CHAPTER 4

FLOW CYTOMETRIC SEXING OF SPIDER SPERM

BRAM VANTHOURNOUT [1]

KIM DESWARTE [2]

HAMIDA HAMMAD [2]

BART LAMBRECHT [2]

FREDERIK HENDRICKX [1,3]

[1] Terrestrial Ecology Unit, Department of Biology, Ghent University, Ledeganckstraat 35, 9000 Ghent, Belgium

[2] Laboratory of Immunoregulation and Department of Respiratory Medicine, University Hospital of Ghent, 185 De Pintelaan, Ghent, B-9000, Belgium

[3] Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium

ABSTRACT

The presence of selfish genetic elements, such as meiotic drive genes, supernumerary chromosomes and endosymbiont bacteria can cause a marked sex ratio distortion in their host. Females of the dwarf spider *Oedothorax gibbosus* have previously been shown to display such female biased sex ratios due to infection with at least two different endosymbiont bacteria. Although pedigree analysis demonstrated that the largest part of sex ratio variation is indeed maternally inherited, additional sex ratio variation was detected, suggesting that additional factors influence the sex ratio in this species. Here, we investigate the presence of sex-chromosome meiotic drive and the occurrence of primary sex ratio compensation by estimating the amount of male and female determining sperm produced by males through the use of flow cytometry. These results show that all investigated males produce equal amounts of male and female determining sperm cells, even when their offspring sex ratio is strongly female biased. Hence, it appears unlikely that a biased production of sperm types contributes to the sex ratio bias in this species. Nevertheless, the use of flow cytometry is put forward as a fast and reliable method to obtain accurate estimates of the proportion of male- and female determining sperm in invertebrates.

1. INTRODUCTION

According to Fisher's theory of sex ratio evolution, frequency dependent selection will increase the fitness of the minority sex and a sex ratio of 50:50 is thus expected to be the evolutionary stable outcome (Fisher, 1930, West, 2009). The evolution of populations and species that produce sex ratios that differ substantially from this ratio therefore intrigued biologists for many decades. One of the causes of such a distortion is the presence of selfish genetic elements, such as genes that exhibit meiotic drive (Lyttle, 1991, Jaenike, 2001), supernumerary chromosomes (Beladjal *et al.*, 2002, Werren and Stouthamer, 2003, Camacho *et a*l., 2011) and infection with endosymbiont bacteria (Stouthamer *et al.*, 1999, Werren *et al.*, 2008, Engelstadter and Hurst, 2009).

In a previous study on the dwarf spider *Oedothorax gibbosus*, we demonstrated a distorting effect caused by maternally inherited endosymbiont bacteria (Vanthournout et al., 2011). Females infected with the endosymbiont *Wolbachia* and a yet unidentified endosymbiont produced sex ratios that were on average more female biased (30% males) compared to those produced by uninfected females (chapter 2). In concordance with sex ratio distortion by endosymbiont bacteria, pedigree analysis revealed that the largest part of the sex ratio variation in this species could indeed be explained by a maternal inheritance pattern. However, significant levels of additional variation were detected suggesting that infection status of females is not the sole factor that determines sex ratio variation in this species (Vanthournout et al. 2011). One such possibility is the presence of suppressor genes, which are thought to evolve by genetic conflict. In accordance with Fisher's sex ratio theory, it is expected that when populations are female biased, strong selection acts on the host nuclear genes that counteract the reproductive effects of the endosymbiont bacterium (Werren and Beukeboom, 1998). Indeed, a strong, direct effect of male nuclear background on the penetrance of the sex ratio effect is observed for this species, which indicates the presence of such suppressor genes (chapter 3). Nevertheless, host resistance to the manipulating effect of the endosymbiont could also evolve by biasing primary sex ratio, i.e. number of males divided by number of total offspring at fertilization, towards males (Werren, 1987).

Alternatively, the observed non-maternal inherited part of sex ratio variation could also be attributed to sex chromosome meiotic drive genes, being the non-Mendelian inheritance of alleles on homologous chromosomes or heteromorphic chromosomes (e.g. sex-chromosomes) resulting in an unequal transmission of both alleles in the offspring (Lyttle, 1991, Jaenike, 2001). In the case of sex-chromosome drive this results in interference of the meiotic division by causing aberrations in the development of sperm cells containing the sex-chromosome subject to drive (Montchamp-Moreau and Joly, 1997, McKee, 1998, Cazemajor *et al.*, 2000, Wilkinson and Sanchez, 2001). As such, the driving sex-chromosome will be overrepresented in the produced gametes.

In the case of *Oedothorax gibbosus*, males are heterogametic with one set of sex-chromosomes X1X2, while in females two sets of chromosomes are present, X1X2 X1X2 (Král & Vanacker, unpublished results). The presence of a driving gene in males on one of the X1X2 chromosomes with deleterious effects on 0-sperm, could lead to a biased production of X1X2-sperm and

therefore to the production of a female distorted sex ratio.

Both hypotheses, sex ratio compensation by biasing the primary sex ratio towards males and the presence of sex-chromosome meiotic drive can be tested by investigating the proportion of both sperm types produced by a male. Traditionally, estimates of the proportion of both types of sperm are obtained through the use of chromosome preparations of sperm nuclei in metaphase and determining the number of male and female determining sperm (Aviles and Maddison, 1991). Unfortunately, the preparation of chromosome slides is highly time consuming and only yields a small sample of investigated sperm. In this paper, we investigate the use of flow cytometry as this allows to examine several thousands of sperm cells in very short time frame. Flow cytometry allows the measurement of physical and chemical characteristics of cells or other biological particles (Shapiro, 2003). These particles pass in a single file through a fluid stream, eventually crossing a laser beam which generates scattering of light and with the use of appropriate dyes emits fluorescence that is picked up by detectors. Multiple parameters can be scored at the same time such as size and granularity of the particle and, in combination with a DNA stain, DNA content of a cell. As the two types of sperm cells contain different sets of chromosomes they can be discerned by quantifying the nuclear DNA content of each sperm cell. By analyzing a large number of sperm nuclei, this produces highly accurate estimates of the proportion of male and female determining sperm that can be used as an indicator for the presence of sex chromosome meiotic drive and to evaluate the hypothesis of sex ratio compensation in this female biased species. We investigate the occurrence of these two phenomena by determining the proportion of male and female yielding sperm in males and relating this to the sex ratio produced by females mated with these males.

2. MATERIAL AND METHODS

2.1 EXPERIMENTAL SETUP

Subadult males were collected in two populations, Damvallei and Walenbos, by means of hand catches and reared in the laboratory under standard conditions. Upon reaching adulthood, male *Oedothorax gibbosus* transfer a droplet of sperm, through the ventral genital pore on the abdomen, onto a silk thread, after which loading of the pedipalps occur. These charged pedipalps, being a modified first pair of legs, function as a sperm reservoir and are subsequently inserted in the genital organ of the female (epigyne) to play an active role in the transfer of sperm (Foelix, 1996; figure 1). Reloading of the palp occurs after mating (pers. obs.).
Upon reaching adulthood, males were mated according to a half-sib breeding design with unrelated females whose offspring were reared till adulthood to determine tertiary sex ratio (number of adult males / total number of adult offspring). After mating, males were anaesthetized using the cold temperature method by placing them in a freezer for approximately one minute. DNA content of individual sperm cells of these paternal males was measured by means of flow cytometry. For a few males, we also measured the DNA content of sperm immediately after reaching adulthood and thus no data of the sex ratio of their offspring is available. DNA of the isolated nuclei was stained with propidium iodide (PI) using the protocol described in Vindelov *et al.* (1983) and Aron *et al.* (2003). Pedipalps were clipped of and ground individually in 200 µl of solution A (trypsin: 1.5 mg/50 ml buffer solution: 200 mg trisodium citrate.$2H_2O$, 104.4 mg spermine tetrahydrochloride, 12.1 mg Tris(hydroxymethyl) aminomethane, 200 µl Igepal and 200 ml dH20; pH 7.6). 10 min later, 150 µl of solution B (25 mg trypsin inhibitor, 5 mg ribonuclease A, 50 ml buffer solution) was added. After 10 min, 150 µl of solution C (20.8 mg propidium iodide, 58 mg spermine tetrahydrochloride, 50 ml buffer solution) cooled in an ice-bath, was added. Preparations were stored at 4 °C for 30-60 min and protected from light by using brown-colored eppendorfs. DNA-content analysis of prepared nuclei was performed on a FACSaria flow cytometer (Argon laser emitting at 488 nm) and the resulting data was processed using FlowJo (Treestar Inc).

Figure 1. Top photo: copulation position of a *tuberosus* male and female *Oedothorax gibbosus*. Picture taken just before male pedipalp insertion into the female genital organ (epigyne). Bottom photo: detail of the male pedipalp and female epigyne, picture taken just before copulation (Dajo Smet).

2.2 STATISTICAL ANALYSIS

Before analysis, dots representing sperm cells were selected by visual inspection of a two dimensional plot depicting propidium iodide (PI) fluorescence intensity, which reflects the DNA content of each particle, and the forward scatter, which is used as a proxy for particle size (figure 2, Shapiro, 2003)

After selecting the appropriate datapoints, estimates of the proportion of 0- and X1X2-containing sperm, p_1 and p_2 respectively, were obtained by fitting a mixture of two normal distributions with unknown means (μ_i), proportion (p_i) and a common unknown variance (σ^2) i.e.

$$X \approx \sum_{i=1}^{2} p_i N(\mu_i, \sigma)$$

where X represents the particle PI fluorescence intensity.
The model was fitted to the data using a Bayesian approach as implemented in Winbugs v.1.4 (Lunn *et al*., 2000). As prior distributions we specified a Dirichlet (1,1) distribution for sampling both proportions, a normal distribution with mean 0 and precision (=1/ σ^2) of 1.0E-6 was chosen as prior distribution for μ_1 and a uniform (0,100) distribution for σ. MCMC chains were run for 10.000 generations, wherein the first 2.000 generations were discarded as burn-in period. This procedure allows obtaining estimates of the produced proportion of each class of sperm cell. From these estimates, we also calculated the mean and coefficient of variation for each peak.

3. RESULTS AND DISCUSSION

Based on the dot plot of nuclei isolated from the pedipalp of an adult male, several dense clouds can clearly be distinguished (figure 2). The two clouds characterized by a high PI fluorescence intensity correspond to a cloud consisting of very small particles (A) and a second cloud consisting of particles with a higher PI fluorescence intensity and a larger forward scatter corresponding to a larger particle size (B). The distribution of the PI fluorescence intensity values in cloud A clearly show a bimodal pattern, suggesting that these correspond to 0-sperm nuclei (left peak) and X1X2-nuclei (right peak), while cloud B corresponds to diploid nuclei that are present in the male palpal organ (figure 2, inset). To confirm that cloud A indeed correspond to sperm nuclei, we also analyzed two male legs (results not shown) and revealed that a cloud of particles with very high PI density and a large size is retained, while a cloud similar to cloud A is absent. This confirms that the bimodal pattern of PI fluorescence intensity values indeed correspond to 0- and X1X2-sperm nuclei.

Based on the results of the statistical analysis, estimates of the proportion of male determining sperm were obtained with an average precision of about 5% (table 1). These values demonstrate that for all investigated males, no evidence was obtained for the production of unequal amounts of X1X2- and 0-sperm. As the sex ratios produced by some of these males clearly deviates from 50% males, there is consequently no relationship between sperm proportion produced by a male and offspring sex ratio. Indeed, a highly distorted sex ratio is often produced by females, even when mated with males producing an equal proportion of sperm types. Also, no differences are observed in the proportion of 0-sperm produced by the two different male morphs and by males originating from the Damvallei and Walenbos population. This is evidence that the female bias in the offspring sex ratio cannot be explained by a biased production of male and female determining sperm. Hence, these data at first indicate that sex chromosome meiotic drive is a less plausible mechanism in the investigated populations. However, to decisively demonstrate the absence of driving sex chromosomes, these results should ideally be complemented with a screening of the number of live and dead sperm as it is possible that the action of the driving gene is situated in the late development of the sperm cell, resulting in the presence of dead spermatozoa in the pedipalp of the male. The combined use of a live cell staining dye (DAPI (4′-6-diamimidino-2-phenylindole), SYBR-14) and a dead cell stain PI (propidium iodide, used in this study) would allow to determine the proportion of live and dead sperm cells (Garner *et al*., 1994, Fry and Wilkinson, 2004, Angelard *et al*., 2008). However, for spiders this remains difficult as live sperm is transferred as packages containing multiple sperm cells (Foelix, 1996, Herberstein *et al*., 2011), which complicates to distinguish multiple peaks for an accurate estimation of the individual proportions.

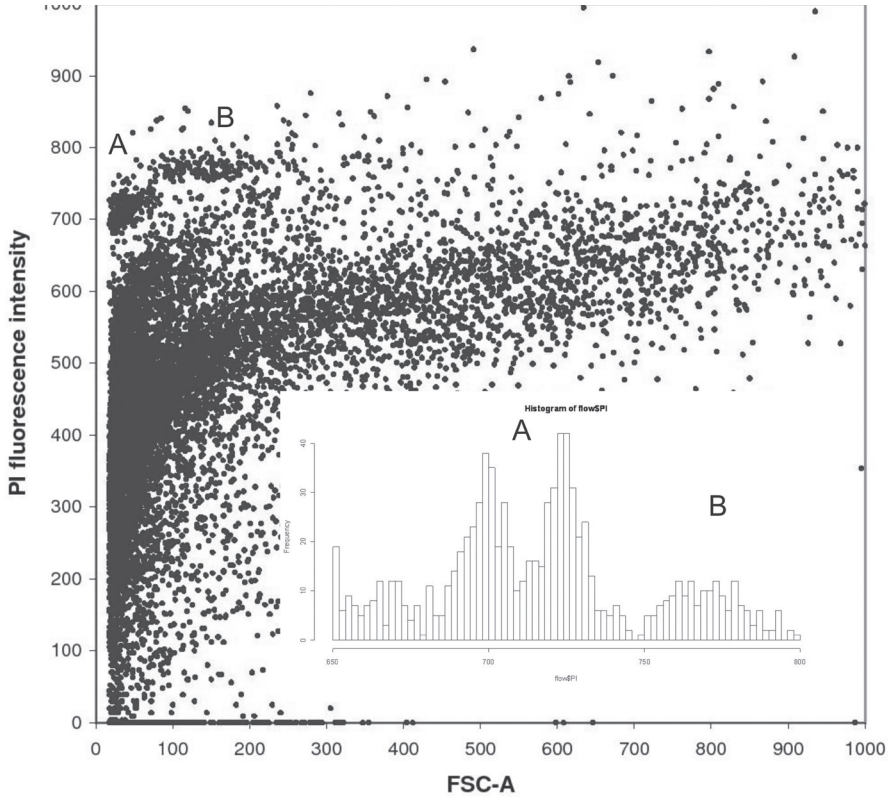


Figure 2: Dotplot of propidium iodide stained nuclei isolated from the pedipalp of an adult male. Two clouds of sperm cells (A) and somatic cells (B) of different DNA content (PI fluorescence intensity) and size (FSC-A) can clearly be distinguished. Inset: flow cytometric DNA histogram of propidium stained nuclei. The two peaks on the left consist of 0 (left)- and X1X2-sperm nuclei (right). The thirth peak represents somatic diploïd nuclei. The large cloud consisting of particles with a large variation in DNA content (PI fluorescence intensity) and size (FSC-A) represents background staining.

The use of flow cytometry in determining the ratio and to isolate sperm types is often adopted in veterinary science and husbandry for sex preselection of mammalian embryos (Garner, 2006) and in plants to investigate the proportion of male and female determining pollen (Stehlik *et al.*, 2007). We show that this technique is also applicable when performed on an invertebrate species as it is possible to discriminate between the sperm cell types based on nuclear DNA content. Moreover, a very large number of sperm nuclei can be processed, resulting in accurate estimates of the number of X1X2 and 0-sperm. The accuracy of the estimated proportions is highly dependent on the difference in DNA content between the two classes of sperm cells which is influenced by the ratio of autosomal chromosomes to sex-chromosomes and the size of the sex-chromosomes. For any given species this difference has to be large enough to allow an irrefutable assignment of nuclei to either class.

Sexing sperm in spider species holds some advantages, as males of spiders store sperm in the two pedipalps prior to fertilization, sperm can readily be obtained by clipping of the pedipalps. Using both pedipalps in the analysis provides two replicate measurements for one individual providing an assessment of the repeatability of the analysis. Next to sperm type analysis, flow cytometry has been used in studies primarily in hymenoptera to investigate brood sex ratio (Aron *et al.*, 2003), individual ploidy level (Boivin and Candau, 2007, Cournault and Aron, 2009) and patterns of sperm number (Cournault and Aron, 2008, Pearcy *et al.*, 2009) and sperm use in ant queens (den Boer *et al.*, 2009). These examples show that flow cytometry is a very versatile technique that can be implemented in a multitude of evolutionary research domains.

Male	Male morph	Pedipalp	Proportion of 0-sperm (Male determining) [Credibility interval]	Total number of counted sperm nuclei	Number of adult males	Number of adult offspring	Sex ratio
D-124	Tub	1 & 2	0.51 [0.47; 0.54]	2286	16	33	0.48
					3	12	0.25
					0	9	0**
D-153	Tub	1	0.51 [0.46; 0.57]	1225	2	43	0.05**
					2	10	0.2
D-85	Tub	1	0.53 [0.49; 0.58]	1119	28	55	0.51
		2	0.54 [0.50; 0.59]	1180	5	10	0.5
D-70	Tub	1	0.53 [0.50; 0.56]	2877	1	14	0.07**
					3	15	0.2*
D-49	Tub	1	0.50 [0.44; 0.58]	1742	14	30	0.47
					14	53	0.26**
					1	7	0.14
D-40	Tub	1	0.50 [0.46; 0.54]	1628	16	26	0.62
					3	15	0.2*
					8	25	0.32
D-87	Tub	1	0.51 [0.47; 0.55]	1214	20	35	0.57
		2	0.51 [0.48; 0.55]	1970			
D-55	Tub	1	0.53 [0.46; 0.59]	840	12	35	0.34
		2	0.53 [0.47; 0.58]	1213			
W-131	Tub	1	0.53 [0.50; 0.57]	1614	3	4	0.75
		2	0.46 [0.41;0.50]	1178	20	37	0.54

W-148	Tub	1	0.53 [0.50; 0.56]	2607	47	76	0.62*
W-132	Tub	1	0.52 [0.48; 0.55]	1514	22	45	0.49
					16	35	0.46
					15	36	0.42
					19	47	0.40
W-221	Tub	1 2	0.51 [0.47; 0.54] 0.54 [0.50;0.57]	1422 1213	33	54	0.61
					5	13	0.38
					29	60	0.48
W-212	Tub	1	0.52 [0.49; 0.55]	2016	22	60	0.37*
		2	0.52 [0.49; 0.55]	2606			
W-102	Tub	1	0.49 [0.42;0.56]	1362	unmated		
		2	0.48 [0.41; 0.55]	1807			
W-111	Tub	1	0.50 [0.46; 0.53]	1445	unmated		
		2	0.47 [0.43; 0.52]	1242			
W-97	Gib	1	0.47 [0.42; 0.54]	803	unmated		
		2	0.48 [0.46; 0.50]	1741			
W-64	Gib	1	0.49 [0.42; 0.56]	1362	unmated		
		2	0.48 [0.41; 0.55]	1807			
D-84	Gib	1	0.49 [0.43; 0.54]	1210	unmated		
		2	0.48 [0.44; 0.52]	1101			
D-125	Gib	1	0.52 [0.48; 0.57]	1390	unmated		
		2	0.53 [0.48; 0.58]	2038			
D-44	Gib	1	0.54 [0.48; 0.60]	1513	unmated		

Table 1. Overview of the proportion of male determining sperm in males of *Oedothorax gibbosus*. Results depict the estimated proportion of 0-sperm (male determining) in one or two pedipalps analyzed for *tuberosus* (Tub) and *gibbosus* (Gib) males originating from two populations Damvallei (D) and Walenbos (W). The sex ratios given represent the proportion of male offspring produced by female mated with the corresponding male. As males were mated with multiple females, multiple sex ratios are given. Sex ratios that differ significantly from a 0.5 sex ratio are indicated with an asterisk (* =< 0.05; ** < 0.01).

AUTHORS' CONTRIBUTION

Bram Vanthournout and Frederik Hendrickx conceived and designed the study. Flow cytometric analysis was performed in association with Ghent University Hospital (UZ Gent) by Bart Lambrecht, Hamida Hammad and Kim Deswarte. Bram Vanthournout and Frederik Hendrickx analyzed the data and wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Kim Deswarte, Dr. Hamida Hammad and Prof. Dr. Bart Lambrecht from Ghent University Hospital for their much appreciated help in the flow cytometric analysis. Pictures were kindly provided by Dajo Smet. Bram Vanthournout is holder of a PhD scholarship grant from the agency for Innovation by Science and Technology (IWT grant n° 73344). Additional financial support was received from the Belgian Science Policy (BELSPO, research project MO/36/O25).

4. REFERENCES

- ANGELARD, C., MONTCHAMP-MOREAU, C. & JOLY, D. 2008. Female-driven mechanisms, ejaculate size and quality contribute to the lower fertility of sex-ratio distorter males in *Drosophila simulans*. *BMC Evolutionary Biology*, 8:326.
- ARON, S., DE MENTEN, L. & VAN BOCKSTAELE, D. 2003. Brood Sex Ratio Determination by Flow Cytometry in Ants. *Molecular Ecology Notes*, 3, 471-475.
- AVILES, L. & MADDISON, W. 1991. When Is the Sex-Ratio Biased in Social Spiders - Chromosome-Studies of Embryos and Male Meiosis in *Anelosimus* Species (Araneae, Theridiidae). *Journal of Arac*hnology, 19, 126-135.
- BELADJAL, L., VANDEKERCKHOVE, T. T. M., MUYSSEN, B., HEYRMAN, J., DE CAESEMAEKER, J. & MERTENS, J. 2002. B-Chromosomes and Male-Biased Sex Ratio With Paternal Inheritance in the Fairy Shrimp *Branchipus Schaefferi* (Crustacea, Anostraca). *Heredity*, 88, 356-360.
- BOIVIN, T. & CANDAU, J.-N. 2007. Flow cytometric analysis of ploidy levels in two seed-infesting *Megastigmus* species: applications to sex ratio and species determination at the larval stage. *Entomologia Experimentalis Et Applicata*, 124, 125-131.
- CAMACHO, J. P. M., SCHMID, M. & CABRERO, J. 2011. B Chromosomes and Sex in Animals. *Sexual Development*, 5, 155-166.
- CAZEMAJOR, M., JOLY, D. & MONTCHAMP-MOREAU, C. 2000. Sex-Ratio Meiotic Drive in *Drosophila Simulans* Is Related to Equational Nondisjunction of the Y Chromosome. *Genetics*, 154, 229-236.
- COURNAULT, L. & ARON, S. 2008. Rapid determination of sperm number in ant queens by flow cytometry. *Insectes Sociaux*, 55, 283-287.
- COURNAULT, L. & ARON, S. 2009. Diploid males, diploid sperm production, and triploid females in the ant *Tapinoma erraticum*. *Naturwissenschaften*, 96, 1393-1400.
- DEN BOER, S. P. A., BAER, B., DREIER, S., ARON, S., NASH, D. R. & BOOMSMA, J. J. 2009. Prudent sperm use by leaf-cutter ant queens. *Proceedings of the Royal Society B-Biological Sciences*, 276, 3945-3953.
- ENGELSTADTER, J. & HURST, G. D. D. 2009. The Ecology and Evolution of Microbes That Manipulate Host Reproduction. *Annual Review of Ecology Evolution and Systematics*, 40, 127-149.
- FISHER, R. A. 1930. *The genetical theory of natural selection.*, Oxford University Press.
- FOELIX, R. F. 1996. *Biology of Spiders*, Oxford University Press.
- FRY, C. L. & WILKINSON, G. S. 2004. Sperm survival in female stalk-eyed flies depends on seminal fluid and meiotic drive. *Evolution*, 58, 1622-1626.
- GARNER, D. L. 2006. Flow cytometric sexing of mammalian sperm. *Theriogenology*, 65, 943-957.
- GARNER, D. L., JOHNSON, L. A., YUE, S. T., ROTH, B. L. & HAUGLAND, R. P. 1994. DUAL DNA STAINING ASSESSMENT OF BOVINE SPERM VIABILITY USING SYBR-14 AND PROPIDIUM IODIDE. *Journal of Andrology*, 15, 620-629.
- HERBERSTEIN, M. E., SCHNEIDER, J. M., UHL, G. & MICHALIK, P. 2011. Sperm dynamics in spiders. *Behavioral Ecology*, 22, 692-695.
- JAENIKE, J. 2001. Sex Chromosome Meiotic Drive. *Annual Review of Ecology and Systematics*, 32, 25-49.

- LUNN, D. J., THOMAS, A., BEST, N. & SPIEGELHALTER, D. 2000. WinBUGS -- a Bayesian modelling framework: concepts, structure, and extensibility. *Statistics and Computing*, 10, 325-337.
- LYTTLE, T. W. 1991. Segregation Distorters. *Annual Review of Genetics*, 25, 511-557.
- MCKEE, B. D. 1998. Pairing sites and the role of chromosome pairing in meiosis and spermatogenesis in male *Drosophila*. *Meiosis and Gametogenesis*, 37, 77-115.
- MONTCHAMP-MOREAU, C. & JOLY, D. 1997. Abnormal spermiogenesis is associated with the X-linked sex-ratio trait in *Drosophila simulans*. *Heredity*, 79, 24-30.
- PEARCY, M., TIMMERMANS, I., ALLARD, D. & ARON, S. 2009. Multiple mating in the ant *Cataglyphis cursor*: testing the sperm limitation and the diploid male load hypotheses. *Insectes Sociaux*, 56, 94-102.
- SHAPIRO, H. M. 2003. *Practical Flow Cytometry*, Wiley-Liss, Inc.
- STEHLIK, I., KRON, P., BARRETT, S. C. H. & HUSBAND, B. C. 2007. Sexing Pollen Reveals Female Bias in a Dioecious Plant. *New Phytologist*, 175, 185-194.
- STOUTHAMER, R., BREEUWER, J. A. J. & HURST, G. D. D. 1999. *Wolbachia pipientis*: Microbial Manipulator of Arthropod Reproduction. *Annual Review of Microbiology*, 53, 71-102.
- VANTHOURNOUT, B., SWAEGERS, J. & HENDRICKX, F. 2011. Spiders do not escape reproductive manipulations by *Wolbachia*. *BMC Evolutionary Biology*, 11:15.
- VINDELOV, L. L., CHRISTENSEN, I. J. & NISSEN, N. I. 1983. A Detergent-Trypsin Method for the Preparation of Nuclei for Flow Cytometric Dna Analysis. *Cytometry*, 3, 323-327.
- WERREN, J. H. 1987. THE COEVOLUTION OF AUTOSOMAL AND CYTOPLASMIC SEX-RATIO FACTORS. *Journal of Theoretical Biology*, 124, 317-334.
- WERREN, J. H., BALDO, L. & CLARK, M. E. 2008. *Wolbachia*: Master Manipulators of Invertebrate Biology. *Nature Reviews Microbiology*, 6, 741-751.
- WERREN, J. H. & BEUKEBOOM, L. W. 1998. Sex Determination, Sex Ratios, and Genetic Conflict. *Annual Review of Ecology and Systematics*, 29, 233-261.
- WERREN, J. H. & STOUTHAMER, R. 2003. PSR (paternal sex ratio) chromosomes: the ultimate selfish genetic elements. *Genetica*, 117, 85-101.
- WEST, S. A. 2009. *Sex Allocation*, Princeton University Press.
- WILKINSON, G. S. & SANCHEZ, M. I. 2001. Sperm development, age and sex chromosome meiotic drive in the stalk-eyed fly, *Cyrtodiopsis whitei*. *Heredity*, 87, 17-24.

“What can I say,
it runs
in the family.”

Oedothorax retusus

CHAPTER 5

ENDOSYMBIONT INDUCED MALE-KILLING IN THE DWARF SPIDER *OEDOTHORAX RETUSUS*

BRAM VANTHOURNOUT [1]

VIKI VANDOMME [1]

FREDERIK HENDRICKX [1,2]

Adapted from Vanthournout, B., Vandomme, V. & Hendrickx, F. Endosymbiont induced male-killing in the dwarf spider *Oedothorax retusus*. Naturwissenschaften (submitted)

[1] **Terrestrial Ecology Unit, Department of Biology, Ghent University, Ledeganckstraat 35, 9000 Ghent, Belgium**

[2] **Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium**

ABSTRACT

Spiders exhibit a remarkable variety of reproductive phenotypes such as parthenogenesis induction and reproductive skew in primary sex ratio. However, the underlying mechanisms remain to this day poorly explored. One of the potential causes of these reproductive alterations is infection with maternally inherited endosymbiont bacteria that alter a mother's offspring sex ratio and hence increasing their own fitness. Although studies show that spiders are infected with several endosymbiont species, it was only recently discovered that endosymbiont bacteria can cause a female sex ratio bias in this order. To explore the distribution and susceptibility to endosymbionts we further investigated the bacterial presence and potential effects in the species *Oedothorax retusus*. Individuals were infected with at least three different endosymbiont species known to cause reproductive alterations in their host i.e. *Wolbachia*, *Rickettsia* and *Cardinium*. Consistent with a bacterial effect the sex ratio bias showed a clear maternal inheritance and treatment with antibiotics reversed the sex ratio to an equal amount of males and females. Female biased clutches were found to exhibit a significantly lower number of hatched spiderlings than unbiased clutches, suggesting the occurrence of male-killing. All females showed infection with *Cardinium*, while only females infected with *Wolbachia* and *Rickettsia* produced a distorted sex ratio. These findings show that effects of endosymbiont bacteria in the order of Araneae could be more widespread than previously assumed and confirm the use of a bacterial model as a mechanistic framework to explain the existence of alternative reproductive phenotypes.

1. INTRODUCTION

Endosymbiont bacteria are maternally inherited micro-organisms that may cause a variety of reproductive alterations in their hosts. Due to their almost exclusively maternal inheritance, induction of parthenogenesis, feminization and male-killing, which all bias the offspring sex ratio towards females, and the occurrence of cytoplasmic incompatibility result in the increase of infected females in the population (Stouthamer *et al*., 1999, Charlat *et al*., 2003, Werren *et al*., 2008, Engelstadter and Hurst, 2009). Owing to their obvious effects on host ecology and reproductive biology, they received increasing attention, resulting in an ever growing number of identified host species infected with microbial reproductive manipulators. Moreover, the combination of screening studies and meta analyses further provides mounting evidence that these endosymbionts are more widespread than previously thought (Goodacre *et al*., 2006, Duron *et al*., 2008a, Hilgenboecker *et al*., 2008). However, knowledge of the phenotypic effects of a large number of such endosymbiont infections remains poorly investigated, leaving major taxonomic groups unexplored. This is particularly the case for the large arthropod group Araneae. Although several studies show that spider species exhibit a high diversity and prevalence of endosymbiont species known to influence their hosts' reproductive biology (Goodacre *et al*., 2006, Baldo *et al*., 2007, Duron *et al*., 2008a, Duron *et al*., 2008b, Martin and Goodacre, 2009, Yun *et al*., 2011), their potential effects are largely unknown. It has been suggested that endosymbionts play a role in influencing offspring sex ratio in the spider *Pityohyphantes phrygianus* (Gunnarsson *et al*., 2009) and only recently it was shown by multiple lines of evidence that the endosymbiont bacterium *Wolbachia* is a causative agent of a female biased sex ratio distortion in the male dimorphic dwarf spider *Oedothorax gibbosus* (Vanthournout *et al*., 2011). Despite these documented cases, it remains at present unknown whether susceptibility to endosymbiont species is confined to only a very limited number of spider species, or whether the effect is more widespread. Moreover, it is striking that sex ratio distorting endosymbiont bacteria are found in a male dimorphic species *Oedothorax gibbosus* (chapter 2 and 3) as a sex ratio bias can have profound effects on the persistence of a male polymorphism in a population (see general introduction). To test the prevalence of the effects of endosymbiont bacteria in spiders, we investigated the presence of such cytoplasmic sex ratio distorting elements in a related species *Oedothorax retusus* (Araneae: Linyphiidae: Erigoninae) that does not exhibit a male polymorphism. This palearctic dwarf spider is found in a variety of mostly wet habitats usually in mosses, grasses and undergrowth and is known to be infected with several endosymbiont species (Goodacre *et al*., 2006), making this a suitable candidate for examination. In this study we investigate the diversity and prevalence of the endosymbiont community in *O. retusus* using endosymbiont specific PCR-assays. To quantify potential effects of each endosymbiont on clutch sex ratio we combined pedigree data resulting from several generations of lab rearing and results from a broad spectrum antibiotic treatment. Furthermore, the phylogenetic position of the identified endosymbiont species was determined and compared to those found in *Oedothorax gibbosus*.

2. MATERIAL AND METHODS

2.1 FIELD COLLECTION, REARING CONDITIONS AND BREEDING DESIGN

Six adult females were collected by means of hand catches at the Damvallei (Belgium) in summer 2010. They were placed individually in plastic vials of 5 cm diameter and 2.5 cm height. Plaster was added to the bottom and moistened with tap water to keep humidity levels at 100%. A piece of moss was added to allow the construction of a functional web. Fruit flies (*Drosophila* sp.) were provided in overabundance and food and humidity levels were checked several times a week. Vials were placed in a climate chamber with a constant temperature of 20°C and a light-dark regime of 16L-8D. Females were allowed to deposit up to three egg sacs before being stored in ethanol. Offspring were reared individually as described above with juvenile spiders receiving collembolans as a food source till the third moult, which was followed by the supply of fruit flies. After the final moult, sex of the spiders was determined by visual inspection using a stereomicroscope. This allowed assessing the tertiary sex ratio (number of adult male offspring / total number of adult offspring). Adult females were then mated with unrelated, lab reared males (n = 22 females) to investigate the inheritance pattern of the sex ratio trait and for the application of antibiotics (see 2.3). Offspring were reared under standard conditions and again tertiary sex ratio was determined. A second generation was reared in the lab to increase sample size and to investigate the underlying mechanism (n = 19 females, see 2.4).

2.2 ENDOSYMBIONT DETECTION AND PHYLOGENETIC RELATIONSHIP

Infection status of the females was investigated by means of PCR-assay for five endosymbionts i.e. *Wolbachia*, *Rickettsia*, *Cardinium*, *Spiroplasma* and *Arsenophonus*, known to potentially cause reproductive alterations in arthropods. Since one female died before being stored in ethanol, three daughters were used in the PCR-assay in order to determine maternal infection status. All three daughters gave consistent results for every endosymbiont tested. Whole spiders were used for DNA extraction using the Nucleospin Tissue kit (©Machery Nagel) following the manufacturers recommended protocol. The following primers were used: (i) 16Swolb99F and 16Swolb99R (Oneill *et al*., 1992) to amplify the 16S ribosomal RNA gene of *Wolbachia*; (ii) *WSP*81F and *WSP*691R (Braig *et al*., 1998) amplifying a part of the cell surface protein coding gene of *Wolbachia* (iii) RICS741F and RCIT1197R (Davis *et al*., 1998, Majerus *et al*., 2000), which amplify a part of the citrate gene of *Rickettsia*; (iv) CLO-f1 and CLO-r1 (Gotoh *et al*., 2007) to selectively amplify a part of the 16S rRNA gene of *Cardinium* (v) SP-ITS-J04 and SP-ITS-N55 (Majerus *et al*., 1999) to selectively amplify the spacer region between the 16S and 23S rRNA genes of *Spiroplasma ixodetis* and (vi) ArsF, ArsF3/ArsR2 to amplify a part of the 16S rDNA of *Arsenophonus nasoniae* (Duron *et al*., 2008a). PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing (54 °C for *Wolbachia*, *Rickettsia* and *Cardinium*; 52 °C for *Arsenophonus* and *Spiroplasma*, 30 s), extension (72 °C, 90 s) and a final extension at 72 °C during 5 min. Electrophoresis was performed on

a 1.5% agarose gel. Gels were stained in a solution of GELRED for approximately 15 min. Bands were visualized by UV-fluorescence. Given that a particular endosymbiont tested positive for all individuals, this ensured that DNA extraction was reliable and no further positive control was used. Prey items can be ruled out as a potential source of *Wolbachia* contamination in the samples as it has previously been shown that our fruit fly and springtail breeding stocks are uninfected (Vanthournout *et al*., 2011). To test the significance of a relationship between endosymbiont infection and sex ratio a generalized linear mixed model was used (Proc GLIMMIX in SAS v.9.1.2.) with endosymbiont presence/absence as fixed effect. Dependence in sex ratio among matrilines was accounted for by including it as a random factor. The obtained estimates of the sex ratios for infected and uninfected females were compared to an even sex ratio using a t-test. Differences in sex ratio between matrilines were analyzed using a chi square test (RxC).

The PCR products were sequenced using BigDye v.1.1 Terminator Sequencing mix and run on an ABI 3710 automated sequencer to check primer specificity and to investigate the presence of different strains. For *Wolbachia*, only the *wsp* gene was sequenced. BLAST searches were used to identify the closest relatives of the obtained endosymbiont sequences. The ClustalW algorithm implemented in MEGA4 (Tamura *et al*., 2007) was used to align the obtained sequences with sequences from other endosymbionts available in Genbank, which mainly originate from the studies reported in (Rowley *et al*., 2004, Goodacre *et al*., 2006, Duron *et al*., 2008b, Wang *et al*., 2010).

Phylogenetic position of the endosymbiont *wsp* (*Wolbachia*), 16S (*Cardinium*) and *citrate* (*Rickettsia*) gene sequences were compared with those found in spider and other host species. For the *wsp* phylogeny, *Wolbachia* supergroup delimitation was used as reported in Rowley *et al*. (2004) and Goodacre *et al*. (2006). A p-distance based Neighbour Joining tree was constructed as implemented in MEGA 5 (Tamura *et al*., 2011). Bootstrap percentage support was calculated for the nodes by generating 10000 bootstrap values.

2.3 ANTIBIOTICS TREATMENT

To test whether administering antibiotics restores an equal sex ratio (Morimoto *et al*., 2006, Gotoh *et al*., 2007), we exposed F1 females from the distorted line (M1; table 1) to the broad spectrum antibiotic tetracycline, which targets endosymbionts from different bacterial families. After reaching adulthood, females were treated by permanently moistening the plaster on the bottom of the vial with the antibiotics solution (0.1%, w/v; 0.002 M). After approximately 7 days females were allowed to copulate with first generation unrelated males. Offspring were reared individually as described above with the continuous use of antibiotic solution.
It has been previously shown that application of antibiotics by moistening plaster is effective in eliminating endosymbionts in the dwarf spider *Oedothorax gibbosus* (Vanthournout *et al*., 2011, chapter 2 and chapter 3) and by spraying the spider in the dwarf spider *Erigone atra* (Goodacre *et al*., 2009). Other F1 females from the distorted matriline were used as a control treatment. Sex ratio and survival of the clutches was determined (number of females: $n = 6$ for both control and tetracycline treatment) and compared between the treatments by means of a generalized

linear mixed model (proc GLIMMIX in SAS v. 9.1.2). To account for dependence in sex ratio among mothers, mother ID was included as random effect.

2.4 MECHANISM

Infection with male-killing endosymbionts typically lowers the clutch size to half the number of clutch sizes produced by uninfected females. Feminization and parthenogenesis induction do not influence clutch size. Based on these properties, it is possible to discriminate among these mechanisms. Therefore, two different approaches were used to investigate the manipulating mechanism. Number of adult offspring and egg sac sex ratio were correlated for all *Wolbachia/Rickettsia* infected (M1, M2; table 1) and all uninfected (M3-M6; table 1) females by means of a Pearson correlation on all clutches weighted for number of adult offspring. In addition a more experimental approach was used: the number of offspring was determined at two different census times, at the egg stage and at hatching from the egg sac. Females that were used to produce the second generation offspring were allowed to oviposit up to three egg sacs before being stored in ethanol. The first egg sac, designated as "after hatching" was allowed to emerge and offspring were reared to adulthood to determine total number of emerged spiderlings and tertiary sex ratio. The second and third egg sac, designated as "before hatching" was stored on ethanol (97%) six days after oviposition to allow sufficient development of the eggs. Afterwards, the proportion of fertile to the total number of eggs produced was determined. This approach is the most favourable to directly link number of emerged spiderlings, egg number and corresponding offspring sex ratio of one female since hatching from the egg and first moult of the spiderlings occurs inside the egg sac. This causes a time lag between hatching of the eggs and emergence of the spiderlings from the egg sac resulting in the inability to discriminate any undeveloped eggs. Total number of emerged spiderlings and number of eggs produced was compared for *Wolbachia/Rickettsia* infection status of the mother, census time (before hatching versus after hatching) and their interaction using a generalized linear mixed model (proc GLIMMIX in SAS v.9.1.2). Identity of the mother was included as random effect to correct for dependence between clutches.

3. RESULTS

3.1 SEX RATIO VARIATION AND ITS RELATIONSHIP WITH ENDOSYMBIONT DETECTION

Field captured females showed a highly significant difference in the sex ratio of their clutches (table 1; df = 5, X^2 = 65.6; P < 0.0001). This difference was primarily attributed to a single female that produced significantly female biased offspring (M1; table 1). Moreover, this bias was maintained by the daughter offspring for at least two subsequent generations, which indicates maternal inheritance. Three females, mated with males originating from this matriline, produced sex ratios that were not significantly different from 0.5 (mean ± SE: 0.54 ± 0.05, $P = 0.22$), indicating that the sex ratio distortion is not heritable through males and confirming the exclusive maternal inheritance. For the other five females, equal amounts of males and females were produced, even in subsequent generations. Individual females were found to be infected with up to three different endosymbionts known to cause reproductive alterations in arthropods. All females were infected with *Cardinium*, while two females were infected with both *Wolbachia* and *Rickettsia*. *Wolbachia/Rickettsia* infection status had a significant effect on the sex ratio produced by a female ($F_{1, 35} = 4.62$; $P = 0.04$) with females infected with *Wolbachia* and *Rickettsia* producing a significantly distorted sex ratio compared to uninfected females (mean ± SE: 0.35 ± 0.06, t_{35}= -2.46, $P = 0.02$; 0.51 ± 0.04, $t_{35} = 0.25$, $P = 0.8$ respectively)

For the two females testing positive for *Wolbachia* infection both the *wsp* and *Wolbachia* specific 16S rRNA primer gave consistent results. Sequencing of the *wsp* primer revealed no individual variation and sequences were easily readable. Blast searches revealed high support with available *Wolbachia* sequences (E-values < 1e-199). The *wsp* sequence [Genbank: JN889706] was most similar with sequences of the spiders *Cybaeus penedentatus* [Genbank: GQ480746], *Araneus diadematus* [Genbank: DQ231505] and *Pityohyphantes phrygianus* [Genbank: DQ231504] and clustered with high support within supergroup B (Additional file 1: Bayesian inference tree of *Wolbachia wsp* sequences).

The same females with *Wolbachia* infection also tested positive for *Rickettsia* with clear bands present. Blast searches confirmed the primer specificity showing homology with *Rickettsia* sequences (E-values < 1e-199). The *Rickettsia* sequence [Genbank: JN889707] showed high similarity with the sequences of the spiders *Oedothorax gibbosus* [Genbank: HQ286292], *Hylaphantes graminicola* [Genbank: DQ231487] and a Theridiidae sp. [Genbank: DQ231486] (Additional file 2: Bayesian inference tree of *Rickettsia* sequences).

For the *Cardinium* endosymbiont clear bands were present for all of the females tested. Alignment of the obtained sequences revealed no individual variation and blast searches yielded high similarity with available *Cardinium* sequences (E-values < 1e-199). Sequences [Genbank: JN889705] were closely related with the *Cardinium* sequence of the spider *Holocnemus pluchei* [Genbank: EU333930] and clustered with high support together with the sequence of the spider *Oedothorax gibbosus* [Genbank: HQ286292] (Additional file 3: Bayesian inference tree of *Cardinium* sequences). Bands were detected for *Arsenophonus* in the two females infected with

Wolbachia and *Rickettsia*. However, sequencing and BLAST searches revealed that these were amplifications of *Rickettsia* and thus constituted false positives. .

Matriline	Endosymbiont infection status			Number of crosses	Number of males	Total number*	Sex ratio	*P*-value**
	Cardinium	*Rickettsia*	*Wolbachia*					
M1	+	+	+	16	123	434	0.29	<.0001
M2	+	+	+	5	62	135	0.46	0.38
M3	+	-	-	6	102	208	0.49	0.8
M4	+	-	-	5	73	165	0.44	0.16
M5	+	-	-	4	88	156	0.56	0.13
M6	+	-	-	5	123	225	0.55	0.18
Tetracycline treatment								
M1				6	71	165	0.43	0.09

Table 1. Sex ratio data and endosymbiont infection status grouped per matriline. * number of adult males + females, ** indicates the probability value of difference from an even sex ratio as calculated by a binomial test.

3.2 ANTIBIOTICS TREATMENT

Treatment with antibiotics significantly affected the tertiary sex ratio produced by a female ($F_{1, 10} = 6.46$; P = 0.03) with control females producing a significantly female biased sex ratio (mean ± SE: 0.21 ± 0.05, $t_{10} = -4.47$, $P = 0.0012$) while tetracycline treatment reverses the sex ratio back to an equal proportion of males and females (mean ± SE: 0.43 ± 0.07, $t_{9.92} = -0.97$, $P = 0.4$). Applying antibiotics did not influence offspring survival as no difference was found ($F_{1, 1} = 2.6$, $P = 0.4$) in survival between tetracycline treated (mean ± SE: 0.93 ± 0.02) and control offspring (mean ± SE: 0.97 ± 0.01).

3.3 MECHANISM

A positive relationship was found between number of adult offspring and egg sac sex ratio with a significant lower proportion of males in smaller egg sacs for *Wolbachia/Rickettsia* infected females (weighted Pearson correlation; $r = 0.57$; $P = 0.005$; figure 1). For uninfected females no correlation could be detected (weighted Pearson correlation; $r = 0.02$; $P = 0.95$; figure 1). Total number of spiderlings is smaller than the total number of eggs produced, irrespective of the *Wolbachia/Rickettsia* infection status of the mother ($F_{1, 31} = 47.58$, $P < 0.0001$). However, significantly fewer spiders emerge as spiderlings when the female is infected with *Wolbachia* and *Rickettsia* (mean ± SE: 19.4 ± 1.8) compared to uninfected mothers

(mean ± SE: 32.8 ± 3.2; $F_{1, 31}$ = 15.32, P = 0.0005; figure 2). *Wolbachia/Rickettsia* infection status has a significant lowering effect on the total number of spiderlings and number of eggs ($F_{1, 13.41}$ = 6.76, P = 0.02). Again, for this subset a significant effect was found of *Wolbachia/Rickettsia* infection status on the offspring sex ratio ($F_{1, 13}$ = 11.67, P = 0.005). *Wolbachia* and *Rickettsia* infected mothers produce a significant female biased sex ratio (mean ± SE: 0.30 ± 0.04; t_{13} =-5.08, P = 0.0002) while uninfected mothers produce an even sex ratio (mean ± SE: 0.48 ± 0.04; $t_{6.78}$ = -0.65, P = 0.54).

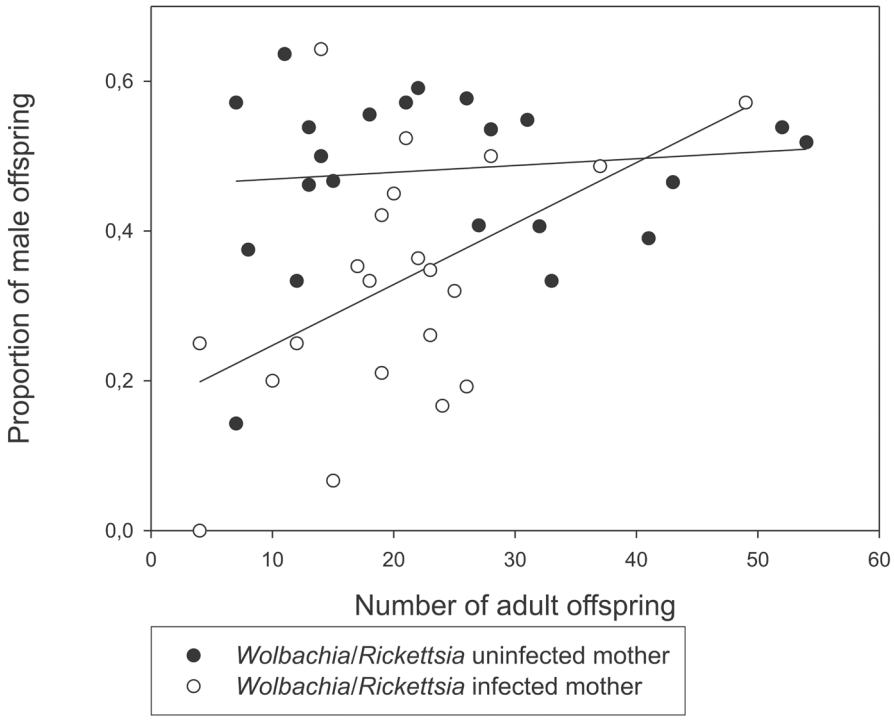


Figure 1. Relationship between number of adult offspring and proportion of male offspring in the egg sac.v
Open circles: *Wolbachia* and *Rickettsia* infected females, filled circles: *Wolbachia* and *Rickettsia* uninfected females. The solid line visualizes the linear correlation.

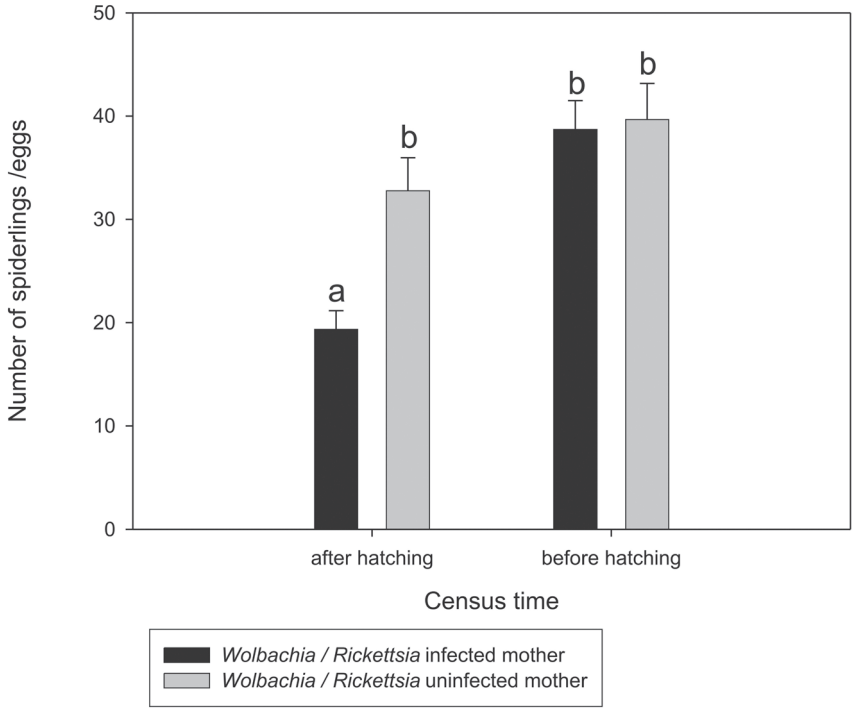


Figure 2. Clutch size number produced by *Wolbachia* and *Rickettsia* infected (black bars) and uninfected (grey bars) females after and before hatching. Bars indicated with the same letter annotation indicate values that are not significantly different.

4. DISCUSSION

In this study we report on the presence of a maternally inherited sex ratio distorting element in the dwarf spider *Oedothorax retusus*. This is deduced from several lines of evidence: (i) one matriline produced significantly female biased offspring sex ratios and several generations of lab rearing reveals that this biased sex ratio trait is maintained when mated with males, originating from even clutches, confirming the exclusive maternal inheritance, (ii) administering antibiotics to this distorted matriline reverses the sex ratio back to an equal proportion of males and females corroborating the bacterial basis and (iii) several endosymbionts known to cause sex ratio biases in their hosts are found to infect this species i.e. *Wolbachia*, *Rickettsia* and *Cardinium*. Differential mortality of the sexes during juvenile development is unlikely to contribute to the sex ratio distortion, as the average juvenile survival is generally high (92%) and highly distorted clutches were found with almost 100% juvenile survival.

All females were found to be infected with *Cardinium* while only two females were infected with *Wolbachia* and *Rickettsia*. As a significant relationship was found between *Wolbachia/ Rickettsia* infection and occurrence of the sex ratio bias, one of these endosymbionts is the most plausible causative agent of the sex ratio distorting effect. However, the relationship between bacterial presence and sex ratio effect is not completely clear-cut. A significant difference in sex ratio exists between the two *Wolbachia* and Rickettsia infected matrilines (table 1; df $= 2$; $X^2 = 14.51$; $P < 0.0002$). This variable pattern of *bacterial* expression could be explained by differences in endosymbiont density levels of which it is known to affect the expression of the reproductive manipulation (Breeuwer and Werren, 1993, Hurst *et al.*, 2000, Bordenstein *et al.*, 2006). Alternatively but not exclusively, the presence of parental suppressor genes could equally produce variation in the expression of the sex ratio trait. Such suppressor genes are commonly expected to evolve in the framework of general sex ratio theory which predicts a 50:50 sex ratio to be the only stable evolutionary strategy from the viewpoint of the host. The discovery of an effect of male nuclear background in the related species *Oedothorax gibbosus* (chapter 3) and in other species harbouring endosymbiont bacteria indeed provides empirical confirmation (Hornett *et al.*, 2006, Majerus and Majerus, 2010b). Performing specific crosses investigating the precise mode of action of a suppressor gene are however necessary to conclusively demonstrate their presence in this species (Majerus and Majerus, 2010b). The combination of these factors does not allow us to assign with high certainty the causative agent for the sex ratio distortion. A first indication of the identity of the sex ratio distorter as well as a possible explanation for the high variation in the sex ratio effect can be obtained by analyzing the different densities of *Wolbachia* and *Rickettsia* using quantitative PCR (Goto et al., 2006). However, obtaining females singly infected with either endosymbiont could lead to more conclusive evidence on the exact roles of each endosymbiont and their possible interaction effects. This can be realized by increasing the sample size of field caught females if natural variation is present between females in infection with either *Wolbachia* or *Rickettsia*. Alternatively, treatment of doubly infected females with low doses of antibiotics (Sasaki et al., 2005) or transfection of endosymbionts through micro-injection (Sasaki et al., 2002) can establish such single infections.

As a clear positive correlation exists between number of adult offspring and egg sac sex ratio with smaller clutches containing significantly less males, killing of males is the most plausible mechanism caused by the endosymbiont infection. This is confirmed by the results from the experiment comparing total number of spiderlings and total number of eggs produced. As expected, spiderling number is significantly smaller than egg number produced for both infected and uninfected mothers. This can be caused by mortality during egg hatching and early juvenile cannibalism occurring inside the egg sac. However, significantly smaller numbers of spiderlings emerge from egg sacs produced by *Wolbachia* and *Rickettsia* infected females. Moreover, the offspring sex ratio is also significantly female biased for these clutches produced by *Wolbachia* and *Rickettsia* infected females compared to uninfected females. As this deviation from an even sex ratio matches the reduction in offspring being produced, this suggests that the offspring which do not emerge from the egg sac are predominantly males. This is again strong evidence for the occurrence of male-killing. Because almost all eggs showed signs of embryonic differentiation (95.7 %, $n = 349$) in the egg sacs of infected mothers the timing of male-killing is most likely situated in the late embryonic development or during hatching.

Establishing the phylogenetic position of the 16S sequence of the *Cardinium* endosymbiont reveals a close relatedness with the sequence of the *Cardinium* endosymbiont infecting the sister species *Oedothorax gibbosus*. This is also the case for the *Rickettsia citrate* gene sequence which is closely related to the sequence of the Rickettsia endosymbiont of *O. gibbosus*. In contrast, a clear dissimilarity is found between the *wsp* sequence of *Wolbachia* as the *O. retusus wsp* sequence clusters within supergroup B while the *wsp* sequence of *O. gibbosus* clusters within supergroup G. Therefore, these data suggest that the *Cardinium* and *Rickettsia* infection predates speciation of the species, followed by independent invasions of different strains of *Wolbachia* in the two species. However, to gain more insight into the routes of infection in the different species and the relatedness between endosymbionts in the *Oedothorax* genus, a more detailed analysis by applying a multilocus comparisons (Baldo *et al*., 2006) would be most suitable.

Although based on a small sample size, it is striking that the prevalence and frequency of endosymbiont species is almost similar to the ones found in *Oedothorax gibbosus* (Vanthournout *et al*., 2011). For the populations investigated, both species seem to be fixed for the *Cardinium* endosymbiont while *Wolbachia* infection shows a more variable pattern with approximately half of the individuals infected. The infection pattern of the *Rickettsia* endosymbiont is different for the two species; *O. gibbosus* seems to be fixed while *O. retusus* shows infection for half of the individuals. Moreover, the extent of the male-killing effect is similar for both systems, with infected females showing a high variation in clutch sex ratio, which is of the same order of magnitude compared to the other *Oedothorax* species (*O. gibbosus*: 0.36 ± 0.04 (Vanthournout *et al*., 2011); *O. retusus*: 0.35 ± 0.06).

This is congruent with the effect of male-killing endosymbionts in several species of ladybirds where similar variation in the production of offspring sex ratios, ranging from all female broods to the production of significant amounts of males, is observed (Hurst *et al*., 1992, Majerus and Majerus, 2010a). In contrast, for butterfly species, infection with male killers has been shown to exhibit a higher level of penetrance with the production of only all female broods (Jiggins *et al*., 2001, Dyson *et al*., 2002). The evolutionary significance of this difference in expression pattern of sex ratio distortion remains to be investigated.

Our findings show that the phenotypic effects of endosymbiont bacteria on reproductive characteristics could be more widespread in the Araneae order. At least for the *Oedothorax* genus it seems that endosymbiont bacteria can have profound effects on their hosts ecology and evolution. This confirms the use of a bacterial model to explain the mechanism of different reproductive phenotypes found in many spider species. Further investigations into the effects in other spider taxa are necessary to determine their general susceptibility to endosymbiont bacteria.

AUTHORS' CONTRIBUTION

Bram Vanthournout and Frederik Hendrickx conceived and designed the study and wrote the manuscript. Bram Vanthournout, Frederik Hendrickx and Viki Vandomme performed the molecular analyses. Bram Vanthournout and Viki Vandomme performed the breeding experiments.

ACKNOWLEDGEMENTS

We thank Lucien Mushimirwa for maintenance of the collembolan and fruit fly breeding stocks and help in rearing the spiders in the lab. Andy Vierstraete performed part of the sequencing analysis. We thank Martijn Vandegehuchte for useful discussions on the Martin sample size.
Bram Vanthournout is holder of a PhD scholarship grant from the agency for Innovation by Science and Technology (IWT grant n° 73344). Additional financial support was received from the Belgian Science Policy (BELSPO, research project MO/36/O25).

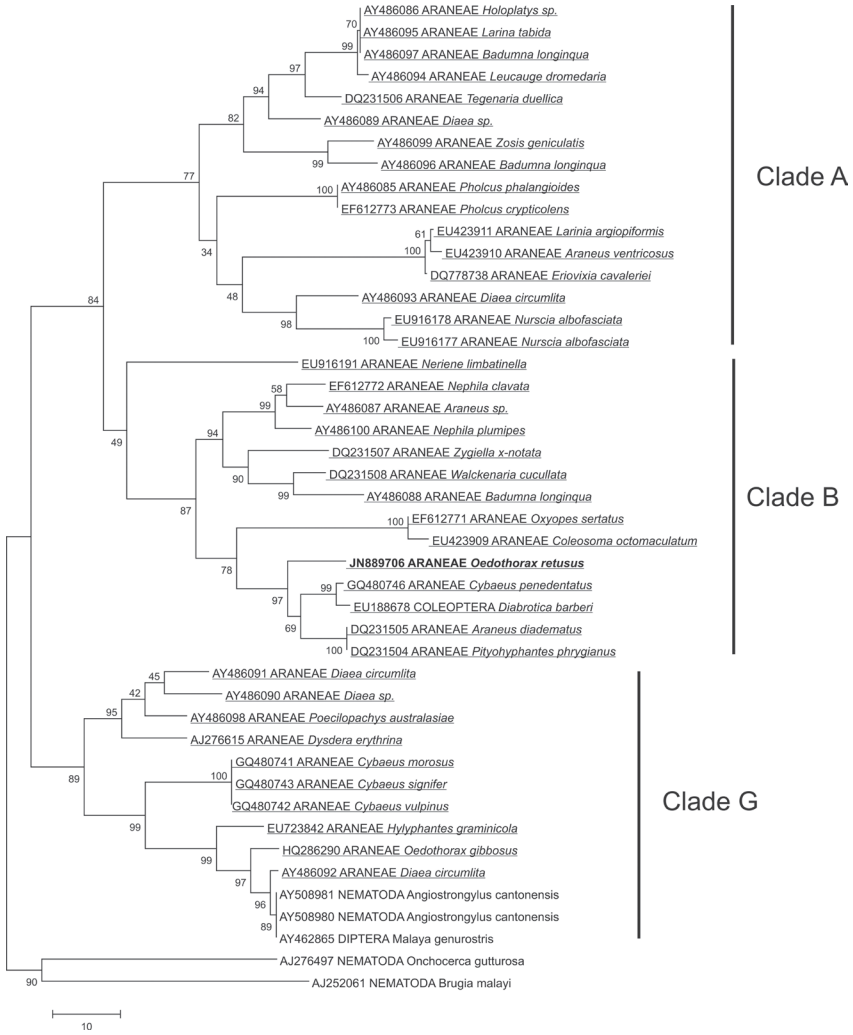
5. REFERENCES

- BALDO, L., AYOUB, N. A., HAYASHI, C. Y., RUSSELL, J. A., STAHLUT, J. K. & WERREN, J. H. 2007. Insight into the routes of *Wolbachia* invasion: high levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity. *Molecular Ecology*, 1-13.
- BALDO, L., HOTOPP, J. C. D., JOLLEY, K. A., BORDENSTEIN, S. R., BIBER, S. A., CHOUDHURY, R. R., HAYASHI, C., MAIDEN, M. C. J., TETTELIN, H. & WERREN, J. H. 2006. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Applied and Environmental Microbiology*, 72, 7098-7110.
- BORDENSTEIN, S. R., MARSHALL, M. L., FRY, A. J., KIM, U. & WERNEGREEN, J. J. 2006. The Tripartite Associations Between Bacteriophage, *Wolbachia*, and Arthropods. *Plos Pathogens*, 2, 384-393.
- BRAIG, H. R., ZHOU, W. G., DOBSON, S. L. & O'NEILL, S. L. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *Journal of Bacteriology*, 180, 2373-2378.
- BREEUWER, J. A. J. & WERREN, J. H. 1993. CYTOPLASMIC INCOMPATIBILITY AND BACTERIAL DENSITY IN NASONIA-VITRIPENNIS. *Genetics*, 135, 565-574.
- CHARLAT, S., HURST, G. D. D. & MERCOT, H. 2003. Evolutionary Consequences of *Wolbachia* Infections. *Trends in Genetics*, 19, 217-223.
- DAVIS, M. J., YING, Z. T., BRUNNER, B. R., PANTOJA, A. & FERWERDA, F. H. 1998. *Rickettsial* relative associated with Papaya bunchy top disease. *Current Microbiology*, 36, 80-84.
- DURON, O., BOUCHON, D., BOUTIN, S., BELLAMY, L., ZHOU, L. Q., ENGELSTADTER, J. & HURST, G. D. 2008a. The Diversity of Reproductive Parasites Among Arthropods: *Wolbachia* Do Not Walk Alone. *BMC Biology*, 6:27.
- DURON, O., HURST, G. D. D., HORNETT, E. A., JOSLING, J. A. & ENGELSTÄDTER, J. 2008b. High incidence of the maternally inherited bacterium *Cardinium* in spiders. *Molecular Ecology*, 1-11.
- DYSON, E. A., KAMATH, M. K. & HURST, G. D. D. 2002. *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera : Nymphalidae): evidence for horizontal transmission of a butterfly male killer. *Heredity*, 88, 166-171.
- ENGELSTADTER, J. & HURST, G. D. D. 2009. The Ecology and Evolution of Microbes That Manipulate Host Reproduction. *Annual Review of Ecology Evolution and Systematics*, 40, 127-149.
- GOODACRE, S. L., MARTIN, O. Y., BONTE, D., HUTCHINGS, L., WOOLLEY, C., IBRAHIM, K., THOMAS, C. F. G. & HEWITT, G. M. 2009. Microbial modification of host long-distance dispersal capacity. *BMC Biology*, 7:32.
- GOODACRE, S. L., MARTIN, O. Y., THOMAS, C. F. G. & HEWITT, G. M. 2006. *Wolbachia* and Other Endosymbiont Infections in Spiders. *Molecular Ecology*, 15, 517-527.
- GOTO, S., ANBUTSU, H. & FUKATSU, T. 2006. Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. *Applied and Environmental Microbiology*, 72, 4805-4810.
- GOTOH, T., NODA, H. & ITO, S. 2007. *Cardinium* Symbionts Cause Cytoplasmic Incompatibility in Spider Mites. *Heredity*, 98, 13-20.

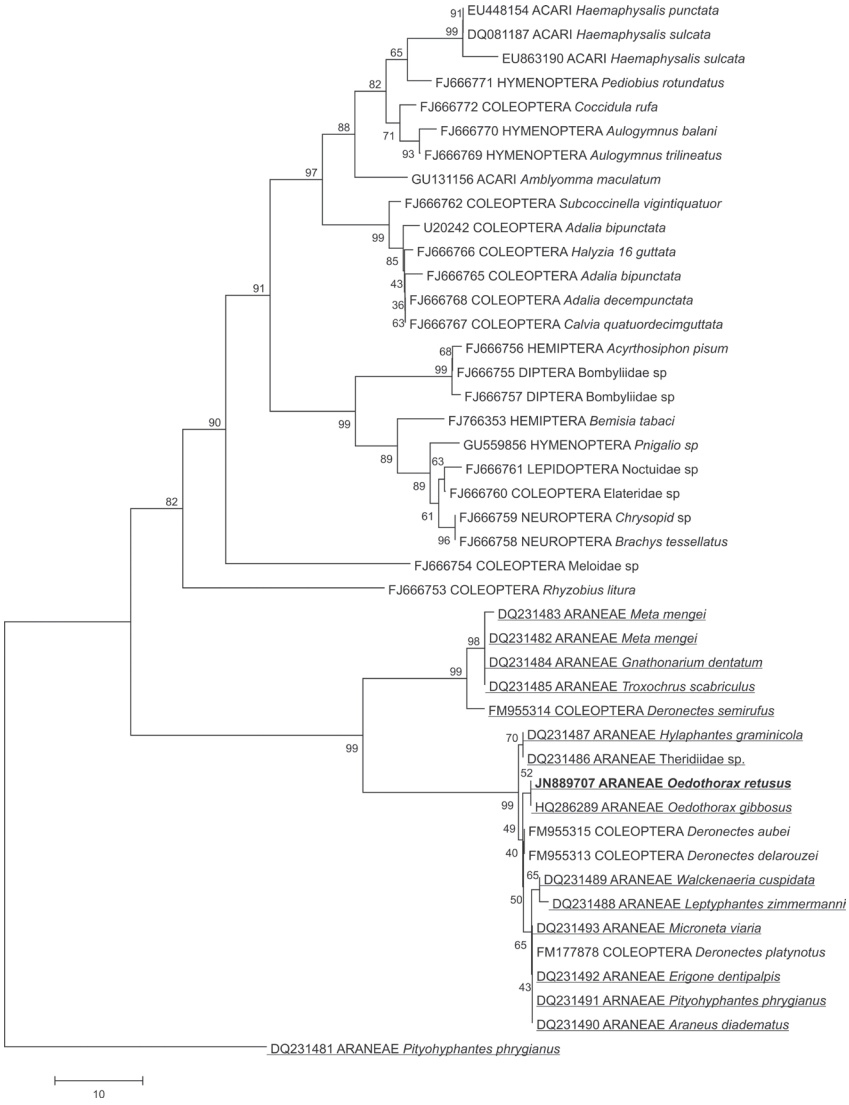
- GUNNARSSON, B., GOODACRE, S. L. & HEWITT, G. M. 2009. Sex Ratio, Mating Behaviour and *Wolbachia* Infections in a Sheetweb Spider. *Biological Journal of the Linnean Society*, 98, 181-186.
- HILGENBOECKER, K., HAMMERSTEIN, P., SCHLATTMANN, P., TELSCHOW, A. & WERREN, J. H. 2008. How Many Species Are Infected With *Wolbachia*? - A Statistical Analysis of Current Data. *Fems Microbiology Letters*, 281, 215-220.
- HORNETT, E. A., CHARLAT, S., DUPLOUY, A. M. R., DAVIES, N., RODERICK, G. K., WEDELL, N. & HURST, G. D. D. 2006. Evolution of Male-Killer Suppression in a Natural Population. *Plos Biology*, 4, 1643-1648.
- HURST, G. D. D., JOHNSON, A. P., VON DER SCHULENBURG, J. H. G. & FUYAMA, Y. 2000. Male-killing *Wolbachia* in *Drosophila*: A temperature-sensitive trait with a threshold bacterial density. *Genetics*, 156, 699-709.
- HURST, G. D. D., MAJERUS, M. E. N. & WALKER, L. E. 1992. CYTOPLASMIC MALE KILLING ELEMENTS IN *ADALIA-BIPUNCTATA* (LINNAEUS) (COLEOPTERA, COCCINELLIDAE). *Heredity*, 69, 84-91.
- JIGGINS, F. M., HURST, G. D. D., SCHULENBURG, J. & MAJERUS, M. E. N. 2001. Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. *Heredity*, 86, 161-166.
- MAJERUS, M. E. N., HINRICH, J., SCHULENBURG, G. V. D. & ZAKHAROV, I. A. 2000. Multiple Causes of Male-Killing in a Single Sample of the Two-Spot Ladybird, *Adalia Bipunctata* (Coleoptera : Coccinellidae) From Moscow. *Heredity*, 84, 605-609.
- MAJERUS, T. M. O. & MAJERUS, M. E. N. 2010a. Discovery and identification of a male-killing agent in the Japanese ladybird *Propylea japonica* (Coleoptera: Coccinellidae). *BMC Evolutionary Biology*, 10:37.
- MAJERUS, T. M. O. & MAJERUS, M. E. N. 2010b. Intergenomic Arms Races: Detection of a Nuclear Rescue Gene of Male-Killing in a Ladybird. *Plos Pathogens*, 6.
- MAJERUS, T. M. O., VON DER SCHULENBURG, J. H. G., MAJERUS, M. E. N. & HURST, G. D. D. 1999. Molecular Identification of a Male-Killing Agent in the Ladybird *Harmonia axyridis* (Pallas) (Coleoptera : Coccinellidae). *Insect Molecular Biology*, 8, 551-555.
- MARTIN, O. Y. & GOODACRE, S. L. 2009. Widespread Infections by the Bacterial Endosymbiont *Cardinium* in Arachnids. *Journal of Arachnology*, 37, 106-108.
- MORIMOTO, S., KURTTI, T. J. & NODA, H. 2006. In Vitro Cultivation and Antibiotic Susceptibility of a Cytophaga-Like Intracellular Symbiote Isolated From the Tick *Ixodes Scapularis*. *Current Microbiology*, 52, 324-329.
- ONEILL, S. L., GIORDANO, R., COLBERT, A. M. E., KARR, T. L. & ROBERTSON, H. M. 1992. 16s Ribosomal-Rna Phylogenetic Analysis of the Bacterial Endosymbionts Associated With Cytoplasmic Incompatibility in Insects. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 2699-2702.
- ROWLEY, S. M., RAVEN, R. J. & MCGRAW, E. A. 2004. *Wolbachia Pipientis* in Australian Spiders. *Current Microbiology*, 49, 208-214.
- SASAKI, T., KUBO, T. & ISHIKAWA, H. 2002. Interspecific transfer of *Wolbachia* between two lepidopteran insects expressing cytoplasmic incompatibility: A *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. *Genetics*, 162, 1313-1319.

- SASAKI, T., MASSAKI, N. & KUBO, T. 2005. *Wolbachia* variant that induces two distinct reproductive phenotypes in different hosts. *Heredity*, 95, 389-393.
- STOUTHAMER, R., BREEUWER, J. A. J. & HURST, G. D. D. 1999. *Wolbachia pipientis*: Microbial Manipulator of Arthropod Reproduction. *Annual Review of Microbiology*, 53, 71-102.
- TAMURA, K., DUDLEY, J., NEI, M. & KUMAR, S. 2007. Mega4: Molecular Evolutionary Genetics Analysis (Mega) Software Version 4.0. *Molecular Biology and Evolution*, 24, 1596-1599.
- TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution, 28, 2731-2739.
- VANTHOURNOUT, B., SWAEGERS, J. & HENDRICKX, F. 2011. Spiders do not escape reproductive manipulations by *Wolbachia*. *BMC Evolutionary Biology*, 11:15.
- WANG, Z. Y., DENG, C., YUN, Y. L., JIAN, C. & PENG, Y. 2010. Molecular detection and the phylogenetics of *Wolbachia* in Chinese spiders (Araneae). *Journal of Arachnology*, 38, 237-241.
- WERREN, J. H., BALDO, L. & CLARK, M. E. 2008. *Wolbachia*: Master Manipulators of Invertebrate Biology. *Nature Reviews Microbiology*, 6, 741-751.
- YUN, Y. L., LEI, C. L., PENG, Y., LIU, F. X., CHEN, J. A. & CHEN, L. B. 2011. *Wolbachia* Strains Typing in Different Geographic Population Spider, *Hylyphantes graminicola* (Linyphiidae). *Current Microbiology*, 62, 139-145.

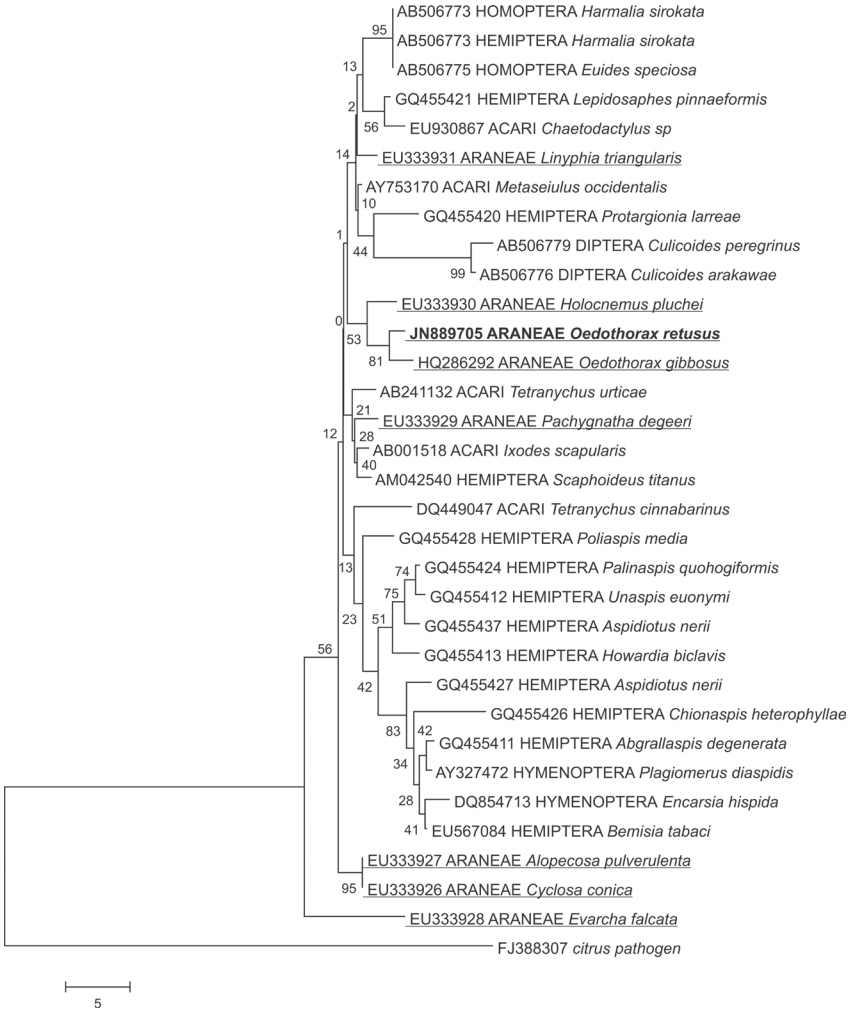
6. ADDITIONAL FILES



Additional file 1. Phylogenetic position of *Wolbachia wsp* sequence of *Oedothorax retusus* [GenBank: JN889706]. Terminal taxa represent host species. A p-distance based Neighbour Joining tree was constructed as implemented in MEGA 5 (Tamura *et al.*, 2011) on a subset of *Wolbachia wsp* sequences available at GenBank, with indication of the major *Wolbachia* supergroups (as reported in Rowley *et al.*, 2004, Goodacre *et al.*, 2006). Percentage bootstrap support was calculated for the nodes. Genbank accession numbers are given in front of the taxonomic group to which the host species belongs. Sequences that originate from spider hosts are underlined. *Oedothorax retusus* is shown in bold.



Additional file 2. Phylogenetic position of the *Rickettsia* (partial *citrate* sequence) endosymbiont of *Oedothorax retusus* [GenBank: JN889707]. Terminal taxa represent host species. A p-distance based Neighbour Joining tree was constructed as implemented in MEGA 5 (Tamura *et al.*, 2011) on a subset of *Rickettsia* sequences available at GenBank. Percentage bootstrap support was calculated for the nodes. Genbank accession numbers are given in front of the taxonomic group to which the host species belongs. Sequences that originate from spider hosts are underlined. *Oedothorax retusus* is shown in bold.



Additional file 3. Phylogenetic position of the *Cardinium* (16S rRNA gene) endosymbiont of *Oedothorax retusus* [GenBank: JN889705]. Terminal taxa represent host species. A p-distance based Neighbour Joining tree was constructed as implemented in MEGA 5 (Tamura *et al.*, 2011) on a subset of *Cardinium* sequences available at GenBank. Percentage bootstrap support was calculated for the nodes. Genbank accession numbers are given in front of the taxonomic group to which the host species belongs. Sequences that originate from spider hosts are underlined. *Oedothorax retusus* is shown in bold.

“A scientist’s aim in a discussion with his colleagues is not to persuade, but to clarify.”

Leó Szilárd

GENERAL DISCUSSION

Investigating the underlying mechanism of sex ratio biases led to the identification of several factors, such as selfish genetic elements, that result in the production of unequal production of male and female offspring (table 1, see also general introduction). As these elements target different aspects of their host reproduction and are transmitted either through males or females, a first indication of the nature of the distorting factor can be found by investigating the direction of this bias and by unraveling the inheritance pattern. Most often heritability values are determined by performing a parent-offspring regression (Falconer and Mackay, 1996) in which the slope of the regression line is an estimate of the heritability of the trait. For sex ratio data a regression can be performed by relating the sex ratio of the clutch of the offspring and the sex ratio of the clutch a parent originated from. Performing this for both male and female parents allows to test whether the sex ratio bias is paternally or maternally inherited (Voordouw *et al.*, 2005). However, this approach is severely limited as it can only incorporate data of two generations in one analysis and therefore reduces the effectiveness in case of large datasets with multiple generations. However, animal models can integrate data obtained from multiple generations by simultaneously incorporating individual phenotypic information of the trait (in casu sex ratio of an individual) and the genetic relatedness of the individuals incorporated in the pedigree. Therefore an animal model approach is most suitable to analyze extensive datasets containing a large amount of information of several generations (Lynch and Walsh, 1998, Wilson *et al.*, 2011). This revealed that the largest source of sex ratio variation originates from the part that is maternally inherited (chapter 1). Additionally, a smaller though significant amount of additional variation was detected, suggesting the occurrence of factors influencing the sex ratio that cannot be explained by a maternal inheritance pattern.

MATERNAL INHERITANCE

ENDOSYMBIONT DETECTION

The observation of a female biased sex ratio in combination with a clear maternal inheritance pattern (Vanthournout *et al*., 2011), strongly suggests that infection with endosymbiont bacteria, rather than the actions of other selfish genetic elements such as sex-chromosome meiotic drive genes (see further) and B-chromosomes (table 1), cause the occurrence of sex ratio distortion. In order to verify endosymbiont presence and explore the diversity of the endosymbiont community in *Oedothorax gibbosus*, individuals of two populations i.e. Damvallei and Walenbos were screened using PCR assays with endosymbiont specific primers in combination with Denaturing Gel Gradient Electrophoresis (DGGE) and cloning (Vanthournout *et al*., 2011 and chapter 2; overview in table 2) of amplicons generated from a PCR based on a general eubacterial primer.

Selfish element	Offspring sex ratio	Inheritance pattern of the sex ratio bias
Meiotic drive genes		
X-chromosome	Female biased sex ratio	Paternal
Y-chromosome	Male biased sex ratio	Paternal
B-chromosomes	Male biased sex ratio	Paternal
Endosymbiont bacteria	Female biased sex ratio	Maternal

Table 1. Overview of the effects of different types of selfish elements on the offspring sex ratio produced by females harbouring this element and indication of the inheritance pattern of the sex ratio bias.

	Damvallei		Damvallei	
	female	male	female	male
Wolbachia	32 (63)	7 (17)	29 (74)	13 (26)
Rickettsia	26 (26)	3 (3)	44 (44)	6 (6)
Cardinium	24 (24)	NA	42 (42)	NA
Spiroplasma	0 (3)	0 (3)	0 (10)	0 (5)
Arsenophonus	0 (14)	NA	0 (27)	NA
Rhabdochlamydia	0 (14)	0 (14)	19 (19)	10 (38)
Orientia	0 (16)	NA	0 (5)	0 (20)
Serratia symbiotica	0 (1)	NA	0 (4)	NA

Table 2. General overview of endosymbiont prevalence (number of infected individuals (total number of screened individuals)) for males and females from two populations of *Oedothorax gibbosus* .

Of the five known endosymbiont genera known to cause reproductive alterations in their host, *Wolbachia*, *Rickettsia* and *Cardinium* were found to infect individuals of *Oedothorax gibbosus*, while no evidence was found of *Spiroplasma* and *Arsenophonus* infection. Also, no evidence was found for infection of *Orientia* and *Serratia* symbiotica, whose effect on the host still remains elusive. However, one additional endosymbiont species, *Rhabdochlamydia*, was detected. Moreover, the four identified endosymbiont species showed marked differences in infection frequency (table 2). *Wolbachia* infection was detected in approximately half of the screened individuals for both the Damvallei and Walenbos population. In contrast, *Rickettsia* and *Cardinium* did not show any variation in infection status as they appear to be fixed in both populations. Interestingly, *Rhabdochlamydia* infection seems to be highly population dependent as it was only detected in individuals of the Walenbos population and not in the Damvallei individuals.

The presence of a *Wolbachia* and *Rickettsia* infection and the absence of *Spiroplasma* is congruent with previous results of an endosymbiont screening of an *Oedothorax gibbosus* female performed by Goodacre (2006). However, as this study did not include *Cardinium* in the analysis, the observation of *Cardinium* infection is the first report for this species. This is in line with the finding that *Cardinium*, although found to also infect other arthropod groups (Zchori-Fein and Perlman, 2004, Hunter and Zchori-Fein, 2006), has a particularly high prevalence in spiders (Duron *et al*., 2008, Martin and Goodacre, 2009). The occurrence of three endosymbionts implies that several spiders are infected with at least three different endosymbiont species which potentially can distort their hosts' reproduction. Moreover, a similar endosymbiont community composition and prevalence has been found in *Oedothorax retusus* (chapter 5) with all individuals showing infection with *Cardinium* and approximately half with *Wolbachia* and *Rickettsia*. However, due to the small sample size, decisive information on the frequency of these endosymbionts in *Oedothorax retusus* could not yet be obtained. Nevertheless, this feature of

triple infection of a single individual in both *Oedothorax* species is one of the few reports in spiders (Goodacre *et al*., 2006).

Investigating the phylogenetic relationship among the sequences obtained from the endosymbionts infecting *O. gibbosus* and *O. retusus* revealed an often remarkably distant relationship. For the 16S sequence of *Cardinium* a high relatedness was detected for the sequences of both species. Similar to *Cardinium*, the *citrate* gene sequence of the *Rickettsia* endosymbiont in *O. retusus* shows a close similarity with the *Rickettsia* endosymbionts infecting *O. gibbosus* and *O. retusus*. Moreover, these sequences cluster within a *Rickettsia* subgroup which seems to be spider specific (Goodacre *et al*., 2006). Contrastingly, the *Wolbachia wsp* sequence of both species showed a large dissimilarity, with the *wsp* sequence of *O. gibbosus* clustering within subgroup G, while the sequence of *O. retusus* clusters within subgroup B. However, the monophyletic origin of supergroup G has recently been questioned as this would constitute a recombination product of supergroups A and B (Baldo and Werren, 2007). High levels of lateral gene transfer have been reported in endosymbiont genomes (Baldo *et al.*, 2006, Moran *et al*., 2008, Ishmael *et al.*, 2009, Klasson *et al.*, 2009), which could imply an overestimation of the evolutionary distance between the two *Wolbachia* infections. However, a four-gamete test of recombination (Librado and Rozas, 2009) revealed no recombination events between the two *Wolbachia* strains. Therefore, these data suggests that *Cardinium* and *Rickettsia* infection was present before both species split, while *Wolbachia* is more likely to have infected both species after their speciation event. However, as this is based on a single gene approach, the robustness of these findings should be tested by incorporating the phylogeny of multiple endosymbiont genes obtained from a larger number of individual spiders.

In chapter 1, a positive relationship was found between *Wolbachia* infection and the occurrence of sex ratio distortion as infected females produced female biased clutches of approximately 30% males, while uninfected females produced equal amounts of male and female offspring. However, clearer evidence of a causative relationship is provided by the application of antibiotics (Stouthamer *et al*., 1999). The bacterial basis of the female bias was confirmed as *Wolbachia* infected females, treated with tetracycline, produced equal sex ratios. The combination of these findings provides strong evidence that at least *Wolbachia* causes a female biased sex ratio in *Oedothorax gibbosus*. To our knowledge this is the first direct evidence of an endosymbiont effect and more specifically of *Wolbachia* on reproduction in the Araneae order. To date, *Wolbachia* is the only endosymbiont which can induce all the four currently known reproductive alterations. Together with a very high infection frequency in arthropods (Hilgenboecker *et al*., 2008) and a large number of identified host species in which *Wolbachia* effects have been demonstrated, it cannot be refuted that *Wolbachia* has a profound effect on arthropod reproduction. However, as argued by Weeks et al. (2002) it is important not to overemphasize the extent of *Wolbachia* effects and caution should be taken in assigning reproductive phenotypes to a particular endosymbiont. Indeed, as shown in chapter 2, a few females also produced an even stronger female biased sex ratios in the absence of *Wolbachia* infection. Again, this bias was found to show an underlying bacterial basis as antibiotic and heat treatment reversed the produced sex ratio back to 0.5. This demonstrates that a similar phenotype can be induced by multiple endosymbiont species in the same population and emphasizes the need for consistent screening.

Currently, the effects of *Rickettsia* and *Cardinium* in *Oedothorax gibbosus* are not known but reproductive effects induced by these endosymbiont species (especially *Rickettsia*) should not be excluded (chapter 2 & 3). The fixed infection frequency in both populations (table 2) suggests that either previous reproductive effects could have been active, or that a current beneficial effect on host fitness allows them to spread in the population. More populations should be sampled to determine if a fixed infection frequency constitutes a species characteristic rather than a population characteristic.

REPRODUCTIVE STRATEGY

Three reproductive strategies employed by endosymbiont bacteria are currently reported to cause a female biased sex ratio in their host, these are parthenogenesis induction, male-killing and feminization (Werren, 1997, Stouthamer *et al*., 1999, Charlat *et al*., 2003, Werren *et al*., 2008, Engelstadter and Hurst, 2009). Parthenogenesis induction is highly unlikely to occur in *Oedothorax gibbosus* as unmated females never produced viable offspring (pers. obs.). Male-killing results in the specific death of male offspring causing a marked reduction in the clutch size compared to uninfected females. In the case of feminization, which turns genetic males into phenotypic females, no effect on the number of offspring is expected. This differential effect on the clutch size produced by infected females allows to discriminate between these strategies. To explore which reproductive manipulation is induced by both *Wolbachia* and the unidentified endosymbiont infection, we related clutch size and clutch sex ratio. A clear positive relationship was detected for both infections, with smaller clutches containing significantly less males. This is consistent with the occurrence of male-killing as the death of male offspring results in a smaller, more female biased clutch. In other arthropod groups, such as ladybirds, butterflies and beetles, the male-killing phenotype was detected by determining the egg hatching rate of infected females. Typically, male-killing lowers the hatching rate to approximately half the hatching rate of uninfected females as males are killed early in embryonic development (Fialho and Stevens, 2000, Jiggins *et al*., 2000, Majerus *et al*., 2000, Jiggins *et al*., 2001). However, in spiders, accurate determination of the hatching rate is difficult as eggs are deposited inside egg sacs hampering visual inspection of egg development. Opening of the egg sac causes the development of fungi resulting in inaccurate estimates of hatching rates (pers. obs.). Moreover, as the first mould of the spiderlings occurs within the egg sac, a time-lag exist between hatching from the egg and emergence of the spiderlings from the egg sac. Therefore, hatching rate can also not be determined after emergence of juvenile spiders as remains of eggs have yet disappeared. A more experimental approach was used in *Oedothorax retusus* (chapter 5) to tackle this problem. The first egg sac of a female was allowed to hatch and offspring were reared till adulthood to determine offspring sex ratio. The second and third egg sac of a female were stored in ethanol (97%) six days after deposition, which enables to discern developed versus undeveloped eggs. In this way, estimates are obtained of the number and sex ratio of hatched juveniles (first egg sac) and the number of viable eggs produced in a clutch (second and third egg sac). Although no significant differences in the number of eggs could be detected between females producing a biased versus unbiased sex ratio, significantly less juvenile spiders hatch from female biased egg sacs, indicating the occurrence of male-killing (chapter 5). Interestingly, all eggs showed evidence of embryonic development,

indicating that the male-killing probably takes place during the late embryonic development. Ideally, this approach should be performed on *Oedothorax gibbosus* to further verify the occurrence of male-killing.

It is interesting to note that both these approaches, i.e. correlating clutch size and clutch sex ratio and determining the number of hatched spiderlings produced by infected and uninfected females, only allow to discern between the male-killing and feminization strategy if both are highly efficient. If male-killing is inefficient, clutch sizes will not be lowered to half of these produced by uninfected females. Similarly, if feminization imposes a cost on the host, a decrease in clutch size is expected as not all males are successfully feminized. The discrimination between both is not always straightforward as demonstrated in the moth *Ostrinia scapulalis* where the production of all female broods was thought to be caused by a feminizing *Wolbachia* endosymbiont (Kageyama *et al*., 2002). However, it was shown that the female bias was in fact caused by lethal feminization of males as sexual mosaic males were produced under reduced *Wolbachia* density (Kageyama and Traut, 2004).

Nevertheless, if feminization would be active in *O. gibbosus* and *O.retusus*, it is expected that some infected females produce male biased clutches after antibiotics treatment as these phenotypic females are in fact genetic males with an underlying male determining sex chromosome combination. No male biased clutches were observed for both *O. gibbosus* and *O. retusus*, indicating that the endosymbiont reproductive strategy is most likely male-killing.

MULTIPLE MALE-KILLERS

Nevertheless, the significant correlation between clutch size and clutch sex ratio in *Oedothorax gibbosus* presents evidence that male-killing is induced by both *Wolbachia* and a putative second endosymbiont. Together with a male-killing endosymbiont found in *Oedothorax retusus* this demonstrates that at least three different male-killers are now recognized in spiders. As some taxonomic groups appear to be particularly prone to infection by male-killers, it has been argued that host life history characteristics might play a role in the invasion of male-killing endosymbionts (Hurst, 1991, Hurst and Majerus, 1993, Majerus and Hurst, 1997). Taxonomic groups such as ladybirds, which show high levels of sibling interaction in early life history stages through egg laying in groups and high levels of sibling cannibalism and strong competition for resources, are shown to be infected with male-killers. As male-killing in itself does not directly increases the proportion of infected females in the population, an indirect increase of the fitness of female offspring is expected through the death of male brothers. This can occur through nutritional advantages by consuming dead males and a decrease in the probability of being cannibalized by other siblings. It has been shown that ladybird larvae, after consuming a sibling egg, show a higher survival time and move faster. This increases the likelihood of finding aphid prey both by the possibility to search a larger area and by experiencing reduced competition. Strikingly, spiders show some ecological similarities with ladybirds in this respect, such as egg laying in egg sacs, high levels of sibling cannibalism and strong local resource competition as spiders are often food-limited (Wise, 2006). Therefore, the same mechanisms as for ladybirds, such as nutritional benefits from consuming dead males and a higher prey availability through

elimination of a potential competitor, could favour male killing in spiders. High levels of sibling cannibalism were found in *Oedothorax gibbosus* in a previous lab study, indicating that direct nutritional benefits through consumption of dead siblings are likely to be present in this species (Vanacker *et al*., 2004a). Moreover, as it was shown that larger offspring are more likely to feed on smaller spiders compared to individuals of the same size, a synergistic effect can be expected. It would be most interesting to further explore the role of host ecology on the persistence of a male-killing phenotype by investigating a larger number of spider species on the presence of male-killing bacteria.

Moreover, it was found that individuals of one population could be infected with two different male-killing endosymbionts (chapter 1 & 2). The presence of multiple male-killers in one population is not fully understood as theoretical models show that stable coexistence is only possible under certain conditions such as spatial division, sibmating and the presence of suppressor genes (see further) (Randerson *et al*., 2000, Groenenboom and Hogeweg, 2002, Dannowski *et al*., 2009). Under the assumptions of infection with a strong and weak male killer, it has been shown that a high spatial structuring of the populations induces the presence of different niches were the stronger and weaker male-killer are differentially selected (Groenenboom and Hogeweg, 2002). Such spatial structuring is thought to be present in *Oedothorax gibbosus* as spiders show a highly clustered distribution within one population. Additionally, this can result in low levels of sibmating which, in combination with high male mating capacity, can produce the conditions for a stable coexistence of a strong and weak male killer (Dannowski *et al*., 2009). Field data of the frequency of sibmating is currently not available for this species. However, it is observed that under laboratory conditions sibmating readily occurs (pers. obs.). Mating experiments revealed a high male mating capacity as viable offspring are produced by up to four females mated with the same male. Additionally, the sperm count of this male (W132) performed by flow cytometry (chapter 4) revealed similar amounts of sperm compared to other males. This indicates that even after four successful matings, males are not sperm depleted.

These models investigating the stable coexistence of such two male-killers assume 100% endosymbiont efficiency, i.e. all infected males die, and varying levels of endosymbiont transmission rate from mother to offspring (Randerson *et al*., 2000, Groenenboom and Hogeweg, 2002, Dannowski *et al*., 2009). These differences in transmission efficiency result in the occurrence of a weaker and stronger male killer within a population. Comparison with the sex ratio data of the *Wolbachia* infected matriline (Wol+) reveals that the sex ratio distortion is significantly more female biased in the matriline infected with an unidentified endosymbiont (Wol-), indicating a higher efficiency of the latter. Indeed, during six generations of lab rearing, low amounts of males were produced by a few females while even sex ratios were produced by only two females in the Wol- matriline. This suggests that *Oedothorax gibbosus* is infected with two male killers which clearly differ in their strength to bias sex ratios. Transmission rate of the *Wolbachia* endosymbiont is considered to be high as all the tested offspring of infected females showed infection (100%, $n = 49$; chapter 1), it can be argued that the transmission rate of the unknown sex ratio distorter is equally high as almost all females produced highly female biased sex ratios for six generations and female offspring, resulting from undistorted clutches, again produced distorted amounts of males and females. This suggest that, in contrast with

the current assumptions of theoretical models, a weaker and stronger male killing phenotype can equally result from a varying endosymbiont efficiency and high transmission rates from mother to offspring. It would be most interesting to investigate the effects of these alternative assumptions on male-killer coexistence by incorporating them into a modelling approach.

RHABDOCHLAMYDIA

Results of the cloning study (chapter 2) revealed the presence of *Rhabdochlamydia* in certain females of the Walenbos population. Subsequent screening of a high number of females showed that *Rhabdochlamydia* infection is highly population dependent as all investigated females of the Walenbos population showed infection, contrasting strongly with the absence of infected females in the Damvallei population (table 2). Bacteria of the Chlamydiales are considered obligate intracellular parasites and are known to infect arthropods (Thao *et al*., 2003, Everett *et al*., 2005, Corsaro and Greub, 2006). In scorpions pathogenic effects are observed caused by a *Porochlamydia* infection in the hepatopancreas (Morel, 1976). *Chlamydia*-like micro-organisms have been observed in spiders (Osaki, 1973), however, as the genus *Rhabdochlamydia* has recently been described and was known to infect only cockroaches and terrestrial isopods (Kostanjsek *et al*., 2004, Corsaro *et al*., 2007), this is the first report in spiders. Pathogenic effects are reported in the cockroach *Blatta orientalis* where *Rhabdochlamydia crassificans* causes abdominal swelling of the host. To date, no effects on host reproduction are documented. However, in the cockroach *Blatta orientalis*, *Rhabdochlamydia* was isolated from both fat body and ovary tissue (Corsaro *et al*., 2007). This association with reproductive tissue is a first indicator of potential reproductive effects. In *Oedothorax gibbosus* the particular pattern of fixed infection in the Walenbos and absence of infection in the Damvallei suggests that *Rhabdochlamydia* could potentially cause the observed sex ratio distortion in interpopulation crosses of Damvallei males and Walenbos females (D x W cross) in the absence of *Wolbachia* (chapter 3). Nevertheless, as further experiments definitely need to be conducted to investigate the role of this endosymbiont, some preliminary experiments were yet performed within the framework of the current study.

First, we attempted to target the *Rhabdochlamydia* infection by means of a specific antibiotic. Offspring resulting from D x W (♂ x ♀) crosses were therefore treated with erythromycin, which is known to target related bacteria of the genus *Chlamydia* (Baron, 1996). As erythromycin is not water soluble, it first has to be resolved in ethanol and subsequently diluted with water to obtain an aqueous solution. This resulted in high levels of juvenile mortality and hence in unreliable estimates of the offspring sex ratio. Pinpointing the optimal concentration of erythromycin by performing a dilution series experiment should be performed to investigate the effects of erythromycin on *Rhabdochlamydia*.

Second, a preliminary transfection experiment was performed by transferring a macerate of an adult Walenbos female infected with *Wolbachia* and *Rhabdochlamydia* to an adult Damvallei female, uninfected with *Wolbachia* and *Rhabdochlamydia* through micro-injection in the region of the epigyne, being the external female genital structure (Sasaki *et al*., 2002). This female was allowed to deposit an egg sac and offspring were reared till adulthood to determine tertiary sex

ratio. A second generation of offspring was obtained by mating female offspring with unrelated males. Interestingly, while originating from a clutch with equal amounts of males and females, this female produced an almost significantly female biased sex ratio of 0.30 ($P = 0.06$; number of offspring = 24). Screening of the injected female gave positive results for the presence of *Rhabdochlamydia* but not for *Wolbachia* infection. Moreover, offspring screening revealed that *Rhabdochlamydia* infection was maintained for at least two generations. This suggests that *Rhabdochlamydia* could possibly induce reproductive effects. However, as these results are currently based on a single female only, these experiments need to be elaborated further.

Third, as *Rhabdochlamydia* infection was only screened in females of the Walenbos population, an additional PCR screening was performed of Walenbos males. This revealed that the prevalence in males (25%; Table 2) was significantly less compared to females, which further suggest that *Rhabdochlamydia* could induce sex specific effects in this species

ADDITIONAL VARIATION

Although results of the animal model analysis revealed that the sex ratio distortion shows a clear maternal inheritance pattern, significant levels of additional variation were detected. This suggests that factors, other than endosymbiont infection, influence sex ratio distortion in this species. In chapters 3 and 4 we investigated the presence of host suppressor genes counteracting the female biasing effects of an endosymbiont infection and the possibility of occurrence of sex-chromosome meiotic drive genes.

SUPPRESSOR GENES

The presence of selfish genetic elements, such as endosymbiont bacteria causing a sex ratio distortion, strongly opposes the Mendelian inheritance pattern of the nuclear genes of the host. Therefore genetic conflict theory predicts strong selection for the evolution of host suppressor genes, counteracting the female biasing effects of endosymbionts (Cosmides and Tooby, 1981, Werren, 1987, Hurst, 1992, Hurst *et al.*, 1996, Werren and Beukeboom, 1998). However, as these counteracting factors are strongly selected for and as such expected to reach high frequencies in a population, demonstrating their presence is challenging. One way to approach this is to perform interpopulation crosses as they allow unlocking the strong association of suppressor genes and endosymbiont bacteria within a population and therefore to disentangle the individual effects of both host and endosymbiont genes. In chapter 3 we performed inter – and intrapopulation crosses using spiders originating from two populations, Damvallei and Walenbos, differing in endosymbiont community (chapter 2). As it was found that Walenbos females produced a significantly more female biased sex ratio compared to Damvallei females, irrespective of the population origin of her mate, these results are in line with genetic conflict theory, i.e. a stronger selection in the Walenbos population to restore an equal sex ratio. This was further confirmed by the observation that Walenbos males performed better in restoring an equal sex ratio compared to Damvallei males, indicating the presence of more effective suppressor genes in the Walenbos populations (chapter 3). In sum, given that these populations do not differ in average sex ratio produced by a female, the occurrence of genetic conflict can strongly mask the effect of endosymbiont bacteria and the presence of suppressors against their effect.

Additionally, field collected males were mated with females originating from two female biased matrilines, one infected with *Wolbachia* (Wol+) and one matriline infected with an unidentified endosymbiont (Wol-). Besides a clear effect of the different matrilines, significant variation was detected in the ability of males to restore an even sex ratio. Moreover, this ability was not consistent as males that were able to restore the sex ratio in one matriline did not necessarily perform equally well for the other matriline. This indicates that the suppressor genes are not equally effective against both the *Wolbachia* endosymbiont and the unidentified endosymbiont distorter. Strikingly, the presence of an endosymbiont specific suppressor gene has been shown to generate a stable male-killer coexistence (Randerson *et al.*, 2000) as selection against one of the endosymbionts allows invasion of the other male-killing endosymbiont and, hence, constitutes a likely mechanism to explain the presence of multiple male-killers in *O. gibbosus* (chapter 2).

The variation among males in their ability to restore sex ratios could have profound implications in the framework of the male dimorphism. In *Oedothorax gibbosus* two types of males can be found (see also general introduction). A *gibbosus* morph, with a hunch on the cephalothorax and an anterior transversal groove, lined with hairs, and a *tuberosus* morph which lacks these cephalic structures. These two types represent alternative mating strategies as it was found that virgin females do not exhibit any preference for either morph, resulting in equal acceptance rates (Vanacker *et al*., 2004b). However, a *gibbosus* male can secure matings with already inseminated females, presumably through providing a substance in the groove which serves as a nuptial gift (Michalik and Uhl, 2011). It has been suggested that the operational sex ratio (ratio of males and females, ready to mate in a population at any given time, Emlen and Oring, 1977, Kvarnemo and Ahnesjo, 1996) could have major implications on the dynamics of a stable male dimorphism in this species (see general introduction). Due to the difference in alternative reproductive strategies of both morphs, only the *gibbosus* morph is accepted by already inseminated females. In combination with the observed patterns of last male sperm priority, this constitutes a fitness advantage for the *gibbosus* male. Therefore, in populations with a more even sex ratio and thus a high number of inseminated females, the relative fitness of *tuberosus* is expected to decrease relatively more compared to *gibbosus*. However, in female biased populations, it is expected that a *tuberosus* male can secure more matings due to its faster juvenile development and longer adult lifespan.

Preliminary theoretical efforts based on evolutionary game theory showed that the male dimorphism would evolve towards a evolutionary stable strategy if both males would be able to adjust their sex ratio, with *tuberosus* males producing a more female biased sex ratio compared to *gibbosus* males (Hendrickx and Mazalov, in prep). This was equally found in previous breeding experiments where females, mated with a *tuberosus* male, tended to produce a more female biased sex ratio (Vanacker, 2005). Merging these previous empirical and theoretical results with the data obtained within the framework of this PhD, the presumed sex ratio adjustment could be attributed to differences in the ability of males to restore an even sex ratio. This hypothesis could, however, only be tested in a very preliminary way. For the experiment investigating the variation between males in restoring an equal sex ratio (chapter 3), field collected males were used of both types of morphs. *Gibbosus* (n = 10) and *tuberosus* (n = 12) males were mated with females from both the Wol- and Wol+ matriline. In line with the above mentioned expectations, females mated with *gibbosus* males tended to produce higher amounts of males compared to *tuberosus* males (figure 1). However, this male morph effect on the offspring sex ratio was not significant ($F_{1, 26.47} = 4.01$, $P = 0.06$).

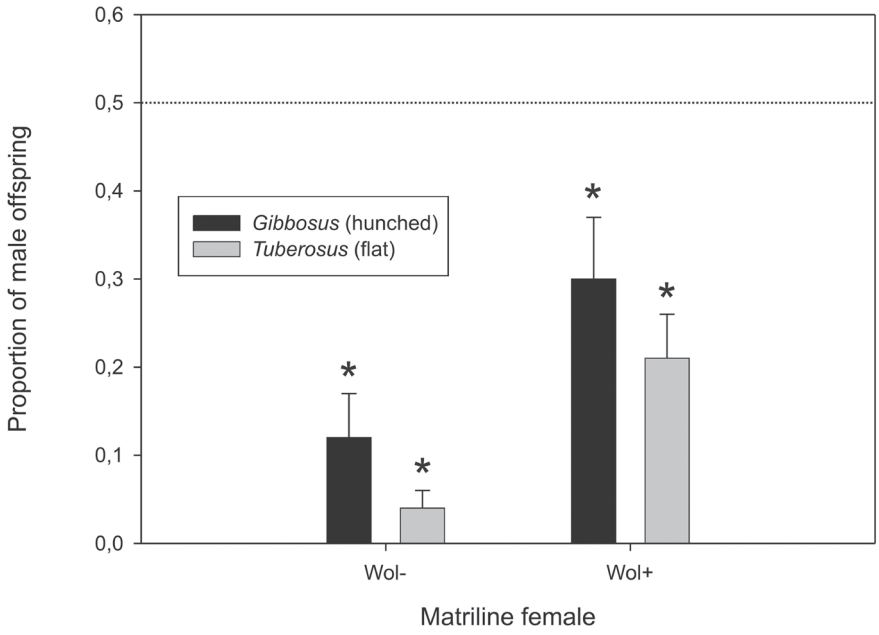


Figure 1. Proportion of male offspring produced by females of two matrilines when mated with different male morphs (Wol+: *Wolbachia* infected, Wol-: unidentified endosymbiont; black bars: *gibbosus* male, grey bars: *tuberosus* male). Striped line depicts an even sex ratio and is given for illustrative purposes, asterisks indicate sex ratios significantly different from 0.5.

These findings suggest that *gibbosus* males could be able to restore an even sex ratio more frequently. Although still premature, genetic linkage of genes coding for the male dimorphism and suppressor genes counteracting the female biasing effect of the endosymbiont could induce such an effect. Repeating this experiment with a higher number of field-collected males and the use of more matrilines would be highly recommended to further explore the potential interactions between male morph and offspring sex ratio. Moreover, the discovery of a similar female biasing factor, i.e. a male-killing endosymbiont, in a related species *Oedothorax retusus* suggests that the occurrence of sex ratio distorting endosymbionts is not limited to male polymorphic spider species.

The animal model employed in chapter 1 allowed to makes estimates of the maternally inherited variation and additional variation. A more complex model was also fitted to this dataset to investigate the existence of host nuclear factors influencing the sex ratio by including a genetic component that contributes to the sex ratio variation. However, this model did not converge and, hence, no accurate estimates could be obtained for this genetic component. This is probably due to the fact that, although based on approximately 4000 spiders, this particular dataset does not contain sufficient half sib families to gain enough statistical power to accurately estimate this genetic variance component. The total dataset obtained from the different breeding designs (chapter 1-4) is based on a parental generation of 275 individuals and several lab reared

generations of which some matrilines have been maintained for six generations. In total, sex ratio data is available based on approximately 11.000 spiders. Therefore, this dataset should be more suitable to investigate the genetic component of sex ratio variation and to quantify the presence of suppressor genes by means of an animal model.

SEX CHROMOSOME MEIOTIC DRIVE

In chapter 4 we investigated to what extent the production of biased amounts of male and female determining sperm influences sex ratio variation in this species. One mechanism that can create such a bias is the occurrence of sex-chromosome meiotic drive causing the non-Mendelian inheritance of a sex-chromosome resulting in its presence in more than half of the produced gametes (Lyttle, 1991, Jaenike, 2001). If a meiotic drive gene is present in *Oedothorax gibbosus* on one of the X-chromosomes, this results in the production of more female determining sperm and hence in the production of a female biased sex ratio. As this feature is paternally inherited (table 1) this could account for the additional variation detected in the animal model analysis (chapter 1).

In concordance with genetic conflict, counteracting effects against the reproductive effects of an endosymbiont infection have been demonstrated in chapter 3. Alternatively, host resistance to a distorted sex ratio could in principle arise equally well from selection on the nuclear genes to compensate the overproduction of the rarer sex (Werren, 1987, Hatcher and Dunn, 1995, West, 2009). To compensate the female biased sex ratio in *Oedothorax gibbosus*, a shift in the primary sex ratio is expected towards males, which can be achieved through the biased production of male determining sperm cells. To date, no empirical evidence has been found of such primary sex ratio compensation.

We used a flow cytometric approach to determine the proportion of sperm types produced by a male to test the above mentioned hypothesis. Flow cytometry allows the discrimination of a large number of sperm cells based on DNA content and therefore assumed to accurately estimate the proportions of male and female determining sperm as information from a very large number of sperm cells per individual can be obtained. However, we could not find any evidence that the production of sperm is biased towards one of the sperm types. This strongly suggests that biased production of sperm cells is absent in the populations examined and therefore no evidence was found of sex chromosome meiotic drive and primary sex ratio compensation. However, the technique of sexing sperm through flow cytometry has proven to be highly efficient as accurate estimates of both sperm types were obtained of a large sample of sperm within a short time-frame. This shows that, although exclusively used in sexing sperm of vertebrates (Garner, 2006), this technique can also be employed on invertebrate sperm and therefore could be implemented in diverse evolutionary domains.

FUTURE PROSPECTS

As deduced from some parts of the general discussion, several aspects of endosymbiont infection in *Oedothorax gibbosus* are in need of more investigative attention in order to gain a more complete understanding of the implications of endosymbiont bacteria in this, as well as other, spider species.

There is a clear need **to fully characterize the endosymbiont community** of *Oedothorax gibbosus*. The recent discovery of new endosymbiont species causing reproductive alterations in their host suggests that the number of currently known endosymbionts is an underestimation of the endosymbiont diversity with more reproductive distorters awaiting discovery. Next generation sequencing would be highly recommended to explore the endosymbiont community as it provides an almost exhaustive assessment of the number of endosymbiont species. For *Oedothorax gibbosus* this could result in the identification of the potential unknown endosymbiont distorter found in chapter 2 and 3.

In line with the previous point, as *Oedothorax gibbosus* shows an exceptionally high diversity of endosymbiont species currently known, this holds a unique opportunity to investigate the **interactions** between members of the **endosymbiont community**. This could be achieved by a determining variation in endosymbiont density through a Q-PCR assay and a combination of specific antibiotics treatments and transfection experiments.

For *Oedothorax gibbsosus*, the occurrence of infection with endosymbionts could have major implications for the persistence of the **male dimorphism** through its influence on frequency dependent selection by altering the operational sex ratio.

As several screening studies show a high prevalence of endosymbionts with known alterations of host reproduction in other spider species, this suggests that more reproductive effects are yet to be discovered. Especially for the male-killing phenotype, three different male-killers have been shown to be present in two spider species, therefore suggesting that this reproductive effect could be more widespread. To determine the extent of endosymbiont induced reproductive alterations in the Araneae order **more spider species** of **different taxonomic groups** should be investigated.

REFERENCES

- BALDO, L., BORDENSTEIN, S., WERNEGREEN, J. J. & WERREN, J. H. 2006. Widespread recombination throughout *Wolbachia* genomes. *Molecular Biology and Evolution*, 23, 437-449.
- BALDO, L. & WERREN, J. H. 2007. Revisiting *Wolbachia* supergroup typing based on *wsp*: spurious lineages and discordance with mlst. *Current Microbiology*, 55, 81-87.
- BARON, S. 1996. Medical Microbiology, Galveston, Texas, University of Texas Medical Branch.
- CHARLAT, S., HURST, G. D. D. & MERCOT, H. 2003. Evolutionary Consequences of *Wolbachia* Infections. *Trends in Genetics*, 19, 217-223.
- CORSARO, D. & GREUB, G. 2006. Pathogenic potential of novel chlamydiae and diagnostic approaches to infections due to these obligate intracellular bacteria. *Clinical Microbiology Reviews*, 19, 283-+.
- CORSARO, D., THOMAS, V., GOY, G., VENDITTI, D., RADEK, R. & GREUB, G. 2007. 'Candidatus *Rhabdochlamydia crassificans*', an intracellular bacterial pathogen of the cockroach *Blatta orientalis* (Insecta : Blattodea). *Systematic and Applied Microbiology*, 30, 221-228.
- COSMIDES, L. M. & TOOBY, J. 1981. CYTOPLASMIC INHERITANCE AND INTRAGENOMIC CONFLICT. *Journal of Theoretical Biology*, 89, 83-129.
- DANNOWSKI, J., FLOR, M., TELSCHOW, A. & HAMMERSTEIN, P. 2009. THE EFFECT OF SIBMATING ON THE INFECTION DYNAMICS OF MALE-KILLING BACTERIA. *Evolution*, 63, 2525-2534.
- DURON, O., HURST, G. D. D., HORNETT, E. A., JOSLING, J. A. & ENGELSTÄDTER, J. 2008. High incidence of the maternally inherited bacterium *Cardinium* in spiders. *Molecular Ecology*, 1-11.
- EMLEN, S. T. & ORING, L. W. 1977. ECOLOGY, SEXUAL SELECTION, AND EVOLUTION OF MATING SYSTEMS. *Science*, 197, 215-223.
- ENGELSTADTER, J. & HURST, G. D. D. 2009. The Ecology and Evolution of Microbes That Manipulate Host Reproduction. *Annual Review of Ecology Evolution and Systematics*, 40, 127-149.
- EVERETT, K. D. E., THAO, M. L., HORN, M., DYSZYNSKI, G. E. & BAUMANN, P. 2005. Novel chlamydiae in whiteflies and scale insects: endosymbionts 'Candidatus *Fritschea bemisiae*' strain Falk and 'Candidatus *Fritschea eriococci*' strain Elm. *International Journal of Systematic and Evolutionary Microbiology*, 55, 1581-1587.
- FALCONER, D. S. & MACKAY, T. F. C. 1996. *Introduction to Quantitative Genetics*, London, Prentice Hall.
- FIALHO, R. F. & STEVENS, L. 2000. Male-Killing *Wolbachia* in a Flour Beetle. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 1469-1473.
- GARNER, D. L. 2006. Flow cytometric sexing of mammalian sperm. *Theriogenology*, 65, 943-957.
- GOODACRE, S. L., MARTIN, O. Y., THOMAS, C. F. G. & HEWITT, G. M. 2006. *Wolbachia* and Other Endosymbiont Infections in Spiders. *Molecular Ecology*, 15, 517-527.
- GROENENBOOM, M. A. C. & HOGEWEG, P. 2002. Space and the persistence of male-killing endosymbionts in insect populations. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 269, 2509-2518.
- HATCHER, M. J. & DUNN, A. M. 1995. EVOLUTIONARY CONSEQUENCES OF CYTOPLASMICALLY INHERITED FEMINIZING FACTORS. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 348, 445-456.

- HILGENBOECKER, K., HAMMERSTEIN, P., SCHLATTMANN, P., TELSCHOW, A. & WERREN, J. H. 2008. How Many Species Are Infected With *Wolbachia*? - A Statistical Analysis of Current Data. *Fems Microbiology Letters*, 281, 215-220.
- HUNTER, M. S. & ZCHORI-FEIN, E. 2006. Inherited Bacteroidetes symbionts in arthropods. *In:* BOURTZIS, K. & MILLER, T. A. (eds.) *Insect Symbiosis*. CRC Press.
- HURST, G. D. D. & MAJERUS, M. E. N. 1993. WHY DO MATERNALLY INHERITED MICROORGANISMS KILL MALES. *Heredity*, 71, 81-95.
- HURST, L. D. 1991. THE INCIDENCES AND EVOLUTION OF CYTOPLASMIC MALE KILLERS. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 244, 91-99.
- HURST, L. D. 1992. INTRAGENOMIC CONFLICT AS AN EVOLUTIONARY FORCE. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 248, 135-140.
- HURST, L. D., ATLAN, A. & BENGTSSON, B. O. 1996. Genetic conflicts. *Quarterly Review of Biology*, 71, 317-364.
- ISHMAEL, N., HOTOPP, J. C. D., IOANNIDIS, P., BIBER, S., SAKAMOTO, J., SIOZIOS, S., NENE, V., WERREN, J., BOURTZIS, K., BORDENSTEIN, S. R. & TETTELIN, H. 2009. Extensive genomic diversity of closely related *Wolbachia* strains. *Microbiology-Sgm*, 155, 2211-2222.
- JAENIKE, J. 2001. Sex Chromosome Meiotic Drive. *Annual Review of Ecology and Systematics*, 32, 25-49.
- JIGGINS, F. M., HURST, G. D. D., JIGGINS, C. D., VON DER SCHULENBURG, J. H. G. & MAJERUS, M. E. N. 2000. The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. *Parasitology*, 120, 439-446.
- JIGGINS, F. M., HURST, G. D. D., SCHULENBURG, J. & MAJERUS, M. E. N. 2001. Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. *Heredity*, 86, 161-166.
- KAGEYAMA, D., NISHIMURA, G., HOSHIZAKI, S. & ISHIKAWA, Y. 2002. Feminizing *Wolbachia* in an Insect, *Ostrinia Furnacalis* (Lepidoptera : Crambidae). *Heredity*, 88, 444-449.
- KAGEYAMA, D. & TRAUT, W. 2004. Opposite sex-specific effects of *Wolbachia* and interference with the sex determination of its host *Ostrinia scapulalis*. *Proc. Biol. Sci.*, 271, 251-258.
- KLASSON, L., WESTBERG, J., SAPOUNTZIS, P., NAESIUND, K., LUTNAES, Y., DARBY, A. C., VENETI, Z., CHEN, L., BRAIG, H. R., GARRETT, R., BOURTZIS, K. & ANDERSSON, S. G. E. 2009. The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 5725-5730.
- KOSTANJSEK, R., STRUS, J., DROBNE, D. & AVGUSTIN, G. 2004. 'Candidatus *Rhabdochlamydia porcellionis*', an intracellular bacterium from the hepatopancreas of the terrestrial isopod *Porcellio scaber* (Crustacea : Isopoda). *International Journal of Systematic and Evolutionary Microbiology*, 54, 543-549.
- KVARNEMO, C. & AHNESJO, I. 1996. The Dynamics of Operational Sex Ratios and Competition for Mates. *Trends in Ecology & Evolution*, 11, 404-408.
- LIBRADO, P. & ROZAS, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451-1452.
- LYNCH, M. & WALSH, B. 1998. *Genetics and analysis of quantitative traits*, Sunderland, MA: Sinauer Associates.
- LYTTLE, T. W. 1991. Segregation Distorters. *Annual Review of Genetics*, 25, 511-557.

- MAJERUS, M. E. N., HINRICH, J., SCHULENBURG, G. V. D. & ZAKHAROV, I. A. 2000. Multiple Causes of Male-Killing in a Single Sample of the Two-Spot Ladybird, *Adalia Bipunctata* (Coleoptera : Coccinellidae) From Moscow. *Heredity*, 84, 605-609.
- MAJERUS, M. E. N. & HURST, G. D. D. 1997. Ladybirds as a model system for the study of male-killing symbionts. *Entomophaga*, 42, 13-20.
- MARTIN, O. Y. & GOODACRE, S. L. 2009. Widespread Infections by the Bacterial Endosymbiont *Cardinium* in Arachnids. *Journal of Arachnology*, 37, 106-108.
- MICHALIK, P. & UHL, G. 2011. Cephalic Modifications in Dimorphic Dwarf Spiders of the Genus *Oedothorax* (Erigoninae, Linyphiidae, Araneae) and Their Evolutionary Implications. *Journal of Morphology*, 272, 814-832.
- MORAN, N. A., MCCUTCHEON, J. P. & NAKABACHI, A. 2008. Genomics and Evolution of Heritable Bacterial Symbionts. *Annual Review of Genetics*, 42, 165-190.
- MOREL, G. 1976. STUDIES ON *POROCHLAMYDIA-BUTHI* G N, SP-N, AN INTRACELLULAR PATHOGEN OF SCORPION *BUTHUS-OCCITANUS*. *Journal of Invertebrate Pathology*, 28, 167-175.
- OSAKI, H. 1973. Electron microscopic observations of *Chlamydia*-like microorganism in hepatopancreas cells of the spider, *Coras luctusosus*. *Acta Arachnol.*, 25, 23-36.
- RANDERSON, J. P., SMITH, N. G. C. & HURST, L. D. 2000. The evolutionary dynamics of male-killers and their hosts. *Heredity*, 84, 152-160.
- SASAKI, T., KUBO, T. & ISHIKAWA, H. 2002. Interspecific transfer of *Wolbachia* between two lepidopteran insects expressing cytoplasmic incompatibility: A *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. *Genetics*, 162, 1313-1319.
- STOUTHAMER, R., BREEUWER, J. A. J. & HURST, G. D. D. 1999. *Wolbachia pipientis*: Microbial Manipulator of Arthropod Reproduction. *Annual Review of Microbiology*, 53, 71-102.
- THAO, M. L., BAUMANN, L., HESS, J. M., FALK, B. W., NG, J. C. K., GULLAN, P. J. & BAUMANN, P. 2003. Phylogenetic evidence for two new insect-associated chlamydia of the family Simkaniaceae. *Current Microbiology*, 47, 46-50.
- VANACKER, D. 2005. *Morphology, function and evolution of the male cephalic structures of the dwarf spider genus Oedothorax*. PhD thesis, Ghent University.
- VANACKER, D., DEROOSE, K., PARDO, S., BONTE, D. & MAELFAIT, J. P. 2004a. Cannibalism and prey sharing among juveniles of the spider *Oedothorax gibbosus* (Blackwall, 1841) (Erigoninae, Linyphiidae, Araneae). *Belgian Journal of Zoology*, 134, 23-28.
- VANACKER, D., HENDRICKX, F., MAES, L., VERRAES, P. & MAELFAIT, J. P. 2004b. Can multiple mating compensate for slower development and shorter adult life in a male dimorphic dwarf spider? *Biological Journal of the Linnean Society*, 82, 269-273.
- VANTHOURNOUT, B., SWAEGERS, J. & HENDRICKX, F. 2011. Spiders do not escape reproductive manipulations by *Wolbachia*. *BMC Evolutionary Biology*, 11:15.
- VOORDOUW, M. J., ROBINSON, H. E. & ANHOLT, B. R. 2005. Paternal Inheritance of the Primary Sex Ratio in a Copepod. *Journal of Evolutionary Biology*, 18, 1304-1314.
- WEEKS, A. R., REYNOLDS, K. T., HOFFMANN, A. A. & MANN, H. 2002. *Wolbachia* Dynamics and Host Effects: What Has (and Has Not) Been Demonstrated? *Trends in Ecology & Evolution*, 17, 257-262.
- WERREN, J. H. 1987. THE COEVOLUTION OF AUTOSOMAL AND CYTOPLASMIC SEX-RATIO FACTORS. *Journal of Theoretical Biology*, 124, 317-334.
- WERREN, J. H. 1997. Biology of *Wolbachia*. *Annual Review of Entomology*, 42, 587-609.

- WERREN, J. H., BALDO, L. & CLARK, M. E. 2008. *Wolbachia*: Master Manipulators of Invertebrate Biology. *Nature Reviews Microbiology*, 6, 741-751.
- WERREN, J. H. & BEUKEBOOM, L. W. 1998. Sex Determination, Sex Ratios, and Genetic Conflict. *Annual Review of Ecology and Systematics*, 29, 233-261.
- WEST, S. A. 2009. *Sex Allocation*, Princeton University Press.
- WILSON, A. J., REALE, D., CLEMENTS, M. N., MORRISSEY, M. M., POSTMA, E., WALLING, C. A., KRUUK, L. E. B. & NUSSEY, D. H. 2011. An ecologist's guide to the animal model (vol 79, pg 13, 2010). *Journal of Animal Ecology*, 80, 1109-1109.
- WISE, D. H. 2006. Cannibalism,food limitation, intraspecific competition and the regulation of spider populations. *Annual Review of Entomology*.
- ZCHORI-FEIN, E. & PERLMAN, S. J. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. *Molecular Ecology*, 13, 2009-2016.

SUMMARY

Sex ratio theory indicates that, given particular assumptions, the production of equal amounts of male and female offspring is the expected evolutionary stable outcome, providing a general theoretical framework for the ubiquity of equal sex ratios. Exceptions to this general rule, i.e. distorted sex ratios, have therefore intrigued biologists for many decades and resulted in the discovery of several mechanisms responsible for male and female biases in offspring production. Yet, the question why sex ratios deviate from this general pattern remains still virtually unexplored for some larger taxonomic groups, such as spiders. Despite the fact that several species are documented to produce distorted sex ratios, the underlying mechanisms are not known. The dwarf spider *Oedothorax gibbosus* holds a unique opportunity to investigate distorted sex ratios in spiders as females show remarkable variation in their offspring sex ratio with some females producing biased amounts of female offspring. This thesis aims to investigate the underlying mechanism by employing an integrative approach of specific breeding designs, pedigree analysis and molecular techniques.

In **chapter 1** we investigated the inheritance pattern of the sex ratio variation as a first step to determine the nature of the sex ratio biasing factor. By fitting an animal model to a pedigree data set containing information of thousands of offspring from multiple generations of lab rearing, it was shown that the largest part of sex ratio variation could be explained by a maternal inheritance pattern. This strongly suggests the involvement of endosymbiont bacteria in causing a sex ratio bias as they are predominantly inherited through females. A subsequent molecular screening of two populations, Damvallei and Walenbos, indeed revealed the presence of several endosymbiont species, i.e. *Wolbachia*, *Rickettsia* and *Cardinium*, known to cause reproductive alterations in their hosts. *Rickettsia* and *Cardinium* were found to be present in all of the screened individuals suggesting that they are fixed in both populations and, hence, unlikely to explain sex-ratio variation. In contrast with this, substantial variation in *Wolbachia* infection status was detected as approximately half of the individuals showed infection. Moreover, *Wolbachia* presence correlated positively with the occurrence of female biased sex ratios of approximately 30% males. The effect of *Wolbachia* was decisively demonstrated by treatment of infected females with antibiotics which resulted in the production of equal amounts of male and female offspring. As significantly less offspring are produced in female biased clutches, male-killing is the most likely reproductive strategy induced by *Wolbachia*.

Although a significant relationship was found between *Wolbachia* infection of a female and the production of female biased sex ratios, some females produced distorted sex ratios in the absence of *Wolbachia* infection. In **chapter 2**, we focus on this apparent additional sex ratio distorting factor by examining the sex ratio variation in females uninfected with *Wolbachia*. Maintaining a lab-reared matriline of one of these females revealed a consistent production of a highly female biased sex ratio in female descendants. Moreover, this sex ratio distortion was only inherited through female offspring as male offspring were not able to induce a female bias in their offspring. As this again suggests the action of endosymbiont bacteria an antibiotics and heat treatment was performed confirming the underlying bacterial basis. Three independent molecular techniques were used to identify this additional endosymbiont. The previously employed endosymbiont specific PCR screening was extended with the use of additional primers for endosymbionts known to infect arthropods. As this technique is limited by primer specificity, two eubacterial 16S rDNA primer based techniques, i.e. cloning of general eubacterial 16S rDNA amplicons and Denaturing Gel Gradient Electrophoresis (DGGE) were used to get a more complete picture of the endosymbiont community. First, these three techniques independently showed that *Wolbachia* is absent in these females producing highly female biased sex ratios while positive results were found using females originating from a matriline known to harbour Wolbachia, demonstrating the effectiveness of these techniques to detect *Wolbachia*. Moreover, *Rickettsia* and *Cardinium* were found in all individuals tested, confirming that they indeed show a fixed prevalence in the populations investigated. The cloning study further revealed the presence of a bacterial species, closely related to *Rhabdochlamydia*, an endosymbiont known to infect arthropods, albeit with no documented effects on host reproduction. A PCR assay with specific primers revealed that *Rhabdochlamydia* infection was population dependent as all females of the Walenbos, but none of the Damvallei population were infected. However, as the results of the molecular techniques did not allow to decisively assign the identity of the endosymbiont distorter, we cannot distinguish between the possibility of one of the current identified endosymbionts causing the female bias or potential effects of an unknown endosymbiont.

The female biased sex ratio, induced by infection with endosymbiont bacteria is expected to strongly select for the evolution of host factors counteracting the endosymbiont effects. In **chapter 3**, we explore the incidence of such intragenomic conflict by investigating the presence of host nuclear genes that suppress the effects of infection with male-killing endosymbionts. We found evidence of such host suppressor genes as inter- and intrapopulation crosses revealed a strong effect of the male nuclear background on the produced sex ratio. If Damvallei males were mated with *Wolbachia* infected females originating from the Walenbos population (D x W cross) , a highly female biased sex ratio was observed. This sex ratio bias was retained when female offspring of this cross were mated with pure Damvallei males, however, when mated with Walenbos males equal amounts of male and female offspring were produced. As an even sex ratio was produced in the first generation after mating, this suggests that the suppressor acts zygotically. Additionally, significant levels of variation were detected between males of the Walenbos population in their ability to restore an equal sex ratio when mated with females from two female-biased matrilines. In sum, these results strongly suggest the presence of host nuclear factors being present in the Walenbos population counteracting the effect of endosymbionts on host reproduction. As the frequency of these suppressor genes seems highly

dependent on population, this suggests local adaptation of populations against the reproductive effects induced by the endosymbiont community. Moreover, no difference in population sex ratio was observed between Damvallei and Walenbos, indicating that the evolution of suppressor genes can mask both the endosymbiont reproductive effects imposed by the endosymbionts.

Though pedigree analysis revealed that a large part of the sex ratio variation could be explained by an inheritance pattern through female offspring, smaller but additional variation was detected which could not be explained by a maternal inheritance pattern. This suggests that additional factors other than endosymbiont bacteria, are present that influence the sex ratio in this species. One potential factor are the effects of host suppressor genes (chapter 3) and, alternatively, the presence of other sex ratio biasing mechanisms, such as the occurrence of sex-chromosome meiotic drive. In *Oedothorax gibbosus* males are the heterogametic sex producing equal male and female determining sperm. However, the presence of driving genes on the sex-chromosomes results in the biased production towards female determining sperm cells, causing a female biased sex ratio. In **chapter 4** we investigate this hypothesis by examining the proportion of male and female determining sperm cells using flow cytometry. Flow cytometry allows the measurement of several cell characteristics including nuclear DNA content of particles crossing a laser beam. Due to the difference in chromosome number between both types of sperm cells, assignment of sperm cells to either class, male or female determining, can be performed with the use of DNA dyes. This analysis revealed that males produce equal proportion of both sperm types, therefore suggesting that biased production of sperm cells due to meiotic drive has at most a minor role in the female biased sex ratio.

In **chapter 5** we explore the sex ratio variation in a related species of dwarf spider *Oedothorax retusus*. It was found that females infected with *Wolbachia* and *Rickettsia* produced significantly female biased sex ratios, indicating that one of these endosymbionts causes a sex ratio distortion in this species. In line with this, antibiotics treatment resulted in the production of equal sex ratios. Fewer spiderlings emerged from egg sacs of infected females compared to uninfected females. As the reduction in spiderling number is of the same order of magnitude as the sex ratio bias, this suggest that mainly males do not hatch. Therefore, male-killing is the most suitable reproductive alteration causing the female bias.

SAMENVATTING

Sex ratio theorie geeft aan dat, onder bepaalde assumpties, de productie van gelijke aantallen mannelijke en vrouwelijke nakomelingen een te verwachten, evolutionair stabiel gevolg is. Dit inzicht voorziet een algemene theorie die het wijdverspreid optreden van gelijke sex ratios voorspelt en verklaart. Het vaststellen van uitzonderingen op deze algemene regel, het optreden van ongelijke sex ratios, is daarom zeer intrigerend en onderzoek naar de oorzaken van een verschuiving naar mannelijke of vrouwelijke nakomelingen leverde verschillende onderliggende mechanismen op. Echter, in sommige grote groepen, zoals spinnen, is de oorzaak van een gedistorseerde sex ratio weinig of niet onderzocht en is het mechanisme onbekend, hoewel sommige spinnensoorten gekend zijn die een sterk gedistorseerde sex ratio vertonen. De bultdwergspin *Oedothorax gibbosus* biedt een unieke gelegenheid om deze problematiek te onderzoeken aangezien vrouwtjes een hoge mate van variatie vertonen in de sex ratio van hun nakomelingen waarbij sommige vrouwtjes een vrouwelijk gedistorseerde sex ratio produceren. Het is dan ook de doelstelling van deze thesis om het onderliggende mechanisme uit te klaren door het geïntegreerd gebruik van een specifieke kweekopzet, stamboomanalyse en moleculaire technieken.

In **hoofdstuk 1** onderzochten we, als een eerste stap in het bepalen van de aard van de sex ratio distorserende factor, het overervingspatroon van de sex ratio variatie. Door het gebruik van een animal model dat de analyse van een stamboom, gebaseerd op de informatie van duizenden nakomelingen, mogelijk maakt, werd het aangetoond dat het grootste deel van sex ratio variatie kan verklaard worden door een overervingspatroon via de moeder. Dit specifiek overervingspatroon suggereert sterk dat endosymbiotische bacteriën de oorzaak zijn van de sex ratio bias aangezien deze enkel overgeërfd worden langs vrouwelijke lijn. Een daaropvolgende moleculaire screening van twee populaties, Damvallei en Walenbos, bevestigde inderdaad de aanwezigheid van verschillende endosymbiotische bacteriën, namelijk *Wolbachia*, *Rickettsia* en *Cardinium*, waarvan effecten op de reproductie van de gastheer gekend zijn. *Rickettsia* en *Cardinium* werden in alle gescreende individuen teruggevonden, suggererend dat ze in beide populaties gefixeerd zijn. Het is daarom onwaarschijnlijk dat deze bacteriële soorten de sex ratio variatie kunnen verklaren. Dit contrasteert sterk met de variatie in infectiestatus teruggevonden bij *Wolbachia* aangezien een infectie werd gedetecteerd bij de helft van de onderzochte individuen. Bovendien werd een positieve correlatie aangetoond tussen de aanwezigheid van *Wolbachia* en het optreden van vrouwelijke gedistorseerde sex ratios van ongeveer 30% mannetjes. Het effect van *Wolbachia* werd overtuigend aangetoond door het behandelen van geïnfecteerde vrouwtjes met antibiotica wat resulteerde in gelijke aantallen van mannelijke en vrouwelijke nakomelingen. Aangezien significant minder nakomelingen werden aangetroffen in vrouwelijk gedistorseerde cocons, is het doden van mannetjes de meest waarschijnlijke reproductieve strategie geïnduceerd door *Wolbachia*.

Hoewel een significante verhouding werd gevonden tussen een *Wolbachia* infectie van een vrouwtje en het produceren van een vrouwelijke gedistorseerde sex ratio, werden er ook gedistorseerde sex ratios aangetroffen in de afwezigheid van *Wolbachia*. In **hoofdstuk 2** focussen we op deze blijkbaar additionele sex ratio distorserende factor door de sex ratio variatie te onderzoeken in vrouwtjes die niet geïnfecteerd zijn met *Wolbachia*. Het in stand houden van een matriline in het labo van één van deze vrouwtjes toonde de consequente productie van vrouwelijke sex ratios aan in vrouwelijke nakomelingen. Bovendien werd deze sex ratio verschuiving enkel overgeërfd via vrouwtjes aangezien mannelijke nakomelingen er niet in slaagden de sex ratio bias door te geven. Dit suggereert opnieuw de invloed van endosymbiotische bacteriën en daarom werd een antibiotica en hitte behandeling uitgevoerd die de onderliggende bacteriële oorzaak bevestigde. Drie onafhankelijke moleculaire technieken werden vervolgens gebruikt om deze additionele endosymbiont te identificeren. De voordien gebruikte, endosymbiont specifieke PCR screening werd uitgebreid door het gebruik van extra primers voor endosymbionten waarvan het aangetoond is dat ze arthropoden infecteren. Aangezien deze techniek sterk gelimiteerd is door de specificiteit van de gebruikte primers werden er twee eubacteriële 16S rDNA primers gebaseerde technieken, clonering van algemene 16S rDNA amplicons en Denaturerende Gel Gradient Electrophorese (DGGE), gebruikt om een beter beeld te verkrijgen van de endosymbiotische gemeenschap.

Deze drie technieken toonden onafhankelijk aan dat *Wolbachia* inderdaad afwezig is in vrouwtjes die een sterk gedistorseerde sex ratio produceren, terwijl positieve resultaten werden gevonden bij vrouwtjes afkomstig van een matrilinie geïnfecteerd met *Wolbachia*. Dit toont duidelijk de efficiëntie aan van de gebruikte moleculaire technieken in het detecteren van *Wolbachia*. Bovendien werd de gefixeerde prevalentie van *Rickettsia* en *Cardinium* in het Walenbos en de Damvallei bevestigd aangezien beide endosymbionten gedecteerd werden in alle gescreende individuen. Het onderzoek via clonering toonde verder de aanwezigheid aan van een endosymbiont, nauw verwant aan *Rhabdochlamydia* waarvan de aanwezigheid in arthropoden aangetoond is zonder enig effect op de reproductie van de gastheer. Een PCR analyse met specifieke primers wees uit dat infectie met *Rhabdochlamydia* populatieafhankelijk is, aangezien alle vrouwtjes uit het Walenbos infectie vertoonden, in tegenstelling tot vrouwtjes uit de Damvallei waarvan geen enkel vrouwtje geïnfecteerd was. Echter, aangezien de resultaten van de moleculaire technieken het niet toelieten om de identiteit van de endosymbiont beslissend toe te kennen, kunnen we op dit moment niet onderscheiden of de vrouwelijke bias veroorzaakt wordt door de huidig geïdentificeerde endosymbionten of door een voorlopig onbekende endosymbiont.

Het wordt verwacht dat de vrouwelijk gedistorseerde sex ratio, geïnduceerd door een infectie met endosymbiotische bacteriën, resulteert in de evolutie van bepaalde factoren in de gastheer die de effecten van een endosymbiotische infectie tegengaan. In **hoofdstuk 3** verkennen we het voorkomen van zo'n intragenomisch conflict door de aanwezigheid te bepalen van gastheer genen die de effecten van het doden van mannetjes door een endosymbionten infectie onderdrukken. Er werd bewijs gevonden voor het optreden van zo'n onderdrukkende genen in de gastheer aangezien een uitgesproken effect werd gedecteerd van de genetische achtergrond van het mannetje op de geproduceerde sex ratio door een vrouwtje. Wanneer mannetjes van de Damvallei gepaard werden met vrouwtjes, geïnfecteerd met *Wolbachia*, uit het Walenbos

(D x W kruising) werd een sterk vrouwelijke bias in de nakomelingen geobserveerd. Deze sex ratio distortie werd behouden wanneer vrouwelijke nakomelingen van deze kruising gepaard werden met pure Damvallei mannetjes, echter, wanneer ze gepaard werden met Walenbos mannetjes, werden gelijke aantallen mannelijke en vrouwelijke nakomelingen geproduceerd. Aangezien een gelijke sex ratio werd geproduceerd in de eerste generatie nakomelingen suggereert dit dat de suppressor werkzaam is in de zygotische fase. Bovendien werd significante variatie gedetecteerd in de mogelijkheid van Walenbos mannetjes om een gelijke sex ratio te herstellen wanneer ze gepaard werden met vrouwtjes van twee vrouwelijk gedistorseerde matrilinies. Samenvattend, deze resultaten wijzen sterk op de aanwezigheid van gastheer factoren in de Walenbos populatie die de effecten van een endosymbionten infectie op de reproductie van de gastheer onderdrukken. Aangezien de frequentie van deze suppressor factoren zeer populatieafhankelijk blijkt, suggereert dit het optreden van lokale adaptatie van populaties tegen de reproductieve effecten geïnduceerd door de endosymbiotische gemeenschap. Bovendien werden er geen verschillen in sex ratio geobserveerd tussen de Damvallei en Walenbos populaties wat er op wijst dat de evolutie van suppressor factoren zowel de reproductieve effecten van endosymbionten als de eigen onderdrukkende effecten kan maskeren.

Hoewel stamboomanalyse uitwees dat het grootste deel van de sex ratio variatie kon verklaard worden via een vrouwelijk overervingspatroon werd er ook lagere, maar significante additionele variatie gedetecteerd dat niet kon verklaard worden door een overerving langs vrouwelijke zijde. Dit suggereert dat bijkomstige factoren, naast endosymbiotische bacteriën, aanwezig zijn die de sex ratio beïnvloeden in deze soort. Eén potentiële factor zijn de effecten van onderdrukkende factoren in de gastheer (hoofdstuk 3) naast de mogelijkheid van het optreden van geslachtschromosoom meiotic drive. In het geval van *Oedothorax gibbosus* zijn mannetjes het heterogametische geslacht dat gelijke aantallen van mannelijk en vrouwelijk determinerende spermacellen produceert. Echter, de aanwezigheid van driving genen op de geslachtschromosomen veroorzaakt een ongelijke productie waardoor meer vrouwelijk determinerende spermacellen geproduceerd worden wat resulteert in een vrouwelijk gedistorseerde sex ratio. We onderzoeken deze hypothese in **hoofdstuk 4** door de proportie aan mannelijk en vrouwelijke determinerende spermacellen te bepalen aan de hand van flow cytometrie. Het gebruiken van flow cytometrie laat het meten van verschillende cel karakteristieken toe, inclusief het DNA gehalte van partikels die een laserstraal passeren. Door de verschillen in chromosoomaantal tussen de twee spermacel types, is het mogelijk om spermacellen toe te kennen aan één van beide klassen, namelijk mannelijk of vrouwelijk determinerend, door het gebruik van een DNA kleuring. Deze analyse toonde aan dat mannetjes een gelijk aantal spermacellen van beide types produceren, wat suggereert dat de overproductie van spermacellen van één type hoogstens een zeer beperkte rol speelt in de gedistorseerde sex ratio.

In **hoofdstuk 5** onderzoeken we de sex ratio variatie in de verwante soort *Oedothorax retusus*. Er werd gevonden dat vrouwtjes, geïnfecteerd met *Wolbachia* en *Rickettsia* een significant vrouwelijk gedistorseerde sex ratio produceerden, wat aantoont dat één van deze endosymbionten de oorzaak is van de geobserveerde sex ratio verschuiving in deze soort. Dit werd bevestigd door het behandelen van vrouwtjes met antibiotica wat het produceren

van gelijke sex ratios tot gevolg had. Een kleiner aantal juveniele spinnen kwam uit eicocons van geïnfecteerde vrouwtjes vergeleken met niet-geïnfecteerde. Aangezien de reductie in aantal juveniele spinnen van dezelfde grootteorde is als de sex ratio bias suggereert dit dat vooral mannetjes niet uit de eicocon komen en, bijgevolg, dat het doden van mannetjes de meest waarschijnlijke reproductieve strategie is.

DANKWOORD

Er werden gedurende dit doctoraat ongeveer 12.000 spinjes van net uit het ei geslopen speldekopje tot een vliegen verslindende predator opgekweekt en die kregen elk ongeveer een 20-tal collembollie en een 15-tal vliegjes als prooi. Bijna overbodig te zeggen dat ik dit en veel andere zaken in het doctoraat verre van alleen heb gedaan, der zijn dan ook heel wat mensen die ik graag zou bedanken.

Fré, nen vré grote merci voor alles, voor het tonen hoe je spinnen vangt en kweekt in het begin van mijn thesis tot de moleculaire technieken aanleren, tot mijn eerste presentatie eens nakijken (Bern, ESA 2008, wat was dat een lelijke presentatie eigenlijk...), tot het eerste artikel submitten, tot de statistische analyses uitleggen en heruitleggen, voor me eigenlijk de manier tot goeie wetenschap bij te brengen. Ik apprecieer het enorm dat ik de vrijheid kreeg om alles op mijn manier te mogen aanpakken met de nodige steun waarvan ik wist dat ik die op ieder moment (bijna letterlijk) kon aanspreken. Het afwerken van het doctoraat op het einde was dan ook op zijn minst een gezamenlijke effort! Maar evengoed voor alles buiten het doctoraat, ne keer één gaan drinken, het amusement op feestjes, recepties, congressen,... Niet te geloven dat het woord greiten je onbekend was!
Daarom, nog één keer, maar dan wel positief: "You won't believe it, that's my supervisor!"

Éééééééééééééééééééééééééééééééééééééééé, Viki, ook een zeer grote bedankt voor alles!
Zeker merci voor al je interesse en al het moleculaire werk (op de vele endosymbionten soorten: Orietia, Orientia, Orientio, Orietio, Serratio, Serrantia, Serrantio, Sorretia, Rickettsio,...)
dat je verzet hebt voor het gibbosus onderzoek en wat heel wat schone resultaten opgeleverd heeft!
Ontelbare spinnen heb je afgezonderd en gecheckt of dat alles nog goe ging, alleen had ik het nie gekund! Zorg goed voor de collembollies! Ook je bijna encyclopedische kennis van waar alles te vinden is in de terec is zeer indrukwekkend en is dan ook de reden waarom ik eerder vroeg: "é viki, waar ligt da?", dan zelfs eens iets te gaan zoeken. Merci!

Een speciaal woord van dank voor Jean-Pierre, de founding father van het gibbosus onderzoek. Bedankt om in het begin van het doctoraat me meteen op sleeptouw te nemen en me de beginselen van wetenschap mee te geven! Ik vraag me vaak af wat je van de resultaten zou gevonden hebben. Zon op al je wegen.

Bedankt aan al de collega's van de Terec en de Limno (jaja, jullie staan er ook bij!) van in de eerste jaren tot helemaal op het einde, heb ik gedurende de vier jaar niet één dag gehad waarop ik met tegenzin naar het werk vertrok. Al moest ik me soms wel haasten om op tijd aan de koffietafel te zitten... Luc, bedankt om de Terec te leiden op zo'n manier waarop iedereen automatisch zijn plaats vindt en zich onmiddelijk thuis voelt! Maurice, Johan, Dries, Carlito, Rein, Eduardo, Lander, Lynda, Val, Toon, Greet, Liesbeth D., Angelica (bedankt voor alle administratieve regelingen!), Bram (als plantenman toch veel respect!), Charlie (kbedenk net dat we toch al aan een gedeelde carrière van bijna 8 jaar zitten, hopelijk doen we der daar nog een paar bij, veel succes!), Joel, Tanja, Katrien, Jeroen (geen schrik Boeye, van de terec heb je lang nog nie alles gezien...), Davy, Tom (slietse dietse), Berber, Debbie, Céline, Lucien (merci voor alle hulp bij de spinnen!), Christoph, Tom C., Helena, Liesbeth W., Nathalie, Arthur, Dieudonné Mbongo, Nele, Pejman, Reza, Sudhi, Leen (rrrustig Leen, rrrustig), Thijs (respect Thijs, respect), Yoeri (postcode van Erwetegem?), Elske, Vanessa, Bob, Hilde, Dirk,... allemaal zeer hartelijk bedankt!!

Ook mijn bureaugenoten mag ik zeker nie vergeten: Hannele, Ellyn, Annelies (Merci voor de zeer belangrijke structuur in deze laatste dagen! Merci pour donner de la structure aux derniers jours!) en Estebano (ondanks je jonge leeftijd ben je toch al een goede wetenschapper!). Als één van de vele lichtpuntjes moet ik toch het voorzitterschap vermelden van het collegiale broeder en zusterschap Brabor Hanmar, merci aan Hanzi (webmaster), Martijn (veldmaarschalk), Boris (penningmeester) en de beëdigde veldmedewerkers Kevin en Viki!

Kortom, alle collega's die ervoor zorgden dat de Terec een zeer leuke plaats is om onderzoek te doen en die ook na de uren voor de nodige ontspanning zorgden, merci!

Ook bedankt aan de vele mensen die zeer veel aan het gibbosus onderzoek bijgedragen hebben, van spinnenvangsten tot verzorgen van de spinnenkweek en moleculair werk: thesisstudenten Janne, Sam, Matthias, Dajo, Jolien en Lien; Joke (merci voor het geduldig uitleggen en de hulp bij de DGGE!), Lut, Léon (Gibbosus onderzoeker van het eerste uur!), Klaas, Andy,... Bedankt aan de mensen van het UZ Gent, Bart Lambrecht, Hamida Hammad en Kim Deswarte, waarvan het enthousiasme om eens een spin door hun machien te jagen zeer aanstekelijk werkte! Een speciale dank voor Danny Vanacker, de ongelooflijk grote hoeveelheid werk die jij verzet hebt, heeft op veel manieren mijn werk veel gemakkelijker gemaakt! Merci!

Graag wil ik ook Silly bedanken (en sjimi, voila, zoals beloofd!) voor haar steun in deze soms wel stressy tijden. Bedankt om er voor me te zijn, je begrip voor een doctoraatsstudent op het einde van zijn doctoraat en goed voor mij te zorgen (kijk al uit naar de volgende lasagne!). Merci, vriendin!

Bedankt aan het mensen van het KBIN voor het gebruik van het moleculair labo en het zorgen voor een zeer fijne werkplek, Wouter, Stefaan, Jeremy, Charlotte,... Bedankt!

I would like to take the opportunity to thank the members of the jury for their time and efforts in commenting on this thesis. I truly feel that, in many ways, the thesis has become a better one because of this. Thank you!

Graag wil ik ook iedereen uit mijn vriendenkring bedanken die nog niet aan bod gekomen zijn, vrienden uit Wijnendale/Torhout, de stadhuiskaarters, vrienden uit Gent, collega-biologen, bosklappers,... Bedankt voor de geestige, ontspannende momenten (ik heb er heel veel aan gehad! Speciale dank gaat uit naar de toog van Gino's!) en de vele steunbetuigingen!

Het wordt soms onderschat hoeveel een warme thuis kan betekenen voor iemand, daarom zou ik graag mijn familie van harte bedanken. Bedankt mams en paps voor jullie vele hulp in kleine en grote dingen en jullie vertrouwen in mijn kunnen. Bedankt! Ook wil ik zeker mijn broers (en vriendin), Arne en Sara en Tim, bedanken voor hun interesse en vele steun!

Ook wil ik zeker en vast Bert bedanken voor de layout van de thesis. Enorm bedankt voor het vele werk, zeker met mijn tijdsschema! Thank god for graphic designers!

Kortom, aan iedereen (hopelijk ben ik niemand vergeten …): Bedankt!