Co-evolution between parthenogenesis-inducing Wolbachia and its hosts.

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Chapter 2

Ecology, *Wolbachia* infection frequency and mode of reproduction in the parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae)

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Published in Molecular Ecology (2010) 19: 1733-1744

Whereas sexual reproduction may facilitate adaptation to complex environments with many biotic interactions, simplified environments are expected to favour asexual reproduction. In agreement with this, recent studies on invertebrates have shown a prevalence of asexual species in agricultural (simplified) but not in natural (complex) environments. We investigated whether the same correlation between reproductive mode and habitat can be found in different populations within one species. The parasitoid wasp *Tetrastichus coeruleus* forms an ideal model to test this question, since it occurs both in natural and agricultural environments. Further, we investigated whether *Wolbachia* infection caused parthenogenesis in female-biased populations.

In contrast to the general pattern, in Dutch and French natural areas we found *Wolbachia*-infected, highly female-biased populations that reproduce parthenogenetically. By contrast, populations on Dutch agricultural fields were not infected with *Wolbachia*, showed higher frequencies of males and reproduced sexually. However, we also found a female-only, *Wolbachia*-infected population on agricultural fields in northeastern USA. All *Wolbachia*-infected populations were infected with the same *Wolbachia* strain.

At this moment, we do not have a convincing explanation for this deviation from the general pattern of ecology and reproductive mode. It may be that asparagus agricultural fields differ from other crop fields in ways that favour sexual reproduction. Alternatively, *Wolbachia* may manipulate life history traits in its host, resulting in different fitness pay-offs in different habitats. The fixation of *Wolbachia*

in the USA populations (where the species was introduced) may be due to founder effect and lack of uninfected, sexual source populations.

Introduction

Sexual reproduction is the predominant mode of reproduction among eukaryotes. This is remarkable given the large fitness disadvantage of sexual reproduction, known as the twofold cost of sex (Maynard Smith 1978). Because an asexual female only produces daughters, all of her offspring will contribute to the next generation, while only half of the offspring (with a 50/50 sex ratio) of a sexually reproducing female will contribute to the next generation. Therefore, an asexual population can grow faster than a sexual population and when they are in competition, the asexual population should outcompete the sexual one (Maynard Smith 1978). Also, asexual females transmit all of their genome to each offspring, while a sexual female transmits only half of her genome to each offspring. Thus, higher mother-offspring relatedness should favour asexual reproduction (Maynard Smith 1978).

Theories that explain the success of sexual reproduction fall into two broad categories (Maynard Smith 1978, Bell 1982). Mutational theories argue that sexual recombination facilitates the shedding of deleterious mutations from the genome, while under asexual reproduction these mutations accumulate and cause a genetic load (Muller 1964, Kimura & Maruyama 1966, Maynard Smith 1978, Crow 1994). Ecological theories suggest that sexual reproduction allows faster adaptation during antagonistic coevolution with other organisms, in particular parasites, but also competitors or predators (Van Valen 1973, Glesener & Tilman 1978, Bell 1985, Hamilton *et al.* 1990, Lively *et al.* 1990). This predicts that the advantage of sex should be smaller in simplified environments with fewer biotic interactions. Asexual reproduction may thus be relatively common in such environments (Glesener & Tilman 1978, Bürger 1999, Haag & Ebert 2004). In support of this idea, recent studies on invertebrates have shown a prevalence of asexual species and populations in agricultural and human-disturbed (simplified) environments, but not in natural (complex) environments (Haack *et al.* 2000, Hoffmann *et al.* 2008, Foucaud *et al.* 2009, Gilabert *et al.* 2009). In order to understand the mechanisms behind this pattern, it is necessary to study populations within one species that occur in different environments. The parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) forms an ideal model to test ecological theories for the maintenance of sexual reproduction, because it occurs both in natural (complex) and in agricultural (simplified) environments.

T. coeruleus is an egg-larval parasitoid of the common asparagus beetle (*Crioceris asparagi*). *C. asparagi* lives on the asparagus plant (*Asparagus officinalis*), which grows on sandy soils, such as coastal dune areas, and as a crop in monoculture on agricultural fields. *A. officinalis* is native to western Asia, Europe and northern Africa and has been cultured for thousands of years (Audas & Heywood 1981, Weeda *et al.* 1991). *C. asparagi* is known as a pest species in asparagus agriculture and *T.*

coeruleus can be used as a biological control agent against these beetles (Capinera & Lilly 1975b). *A. officinalis* has been introduced to the United States for culturing (Weeda *et al.* 1991) and in 1859 *C. asparagi* was first noticed in northeastern United States (Capinera & Lilly 1975a). Later, probably already in 1863, but certainly in 1909, *T. coeruleus* also was recorded and was noticed to control the asparagus beetle population by feeding on and ovipositing in the eggs of *C. asparagi* (Russell & Johnston 1912, Johnston 1915, Capinera & Lilly 1975a, 1975b). Russell & Johnston (1912) and Johnston (1915) only recorded females of *T. coeruleus* in the population, both in the field and when they were reared in the lab for multiple generations, showing that *T. coeruleus* occurs in parthenogenetic populations on agricultural fields in northeastern United States.

Asexual reproduction in invertebrates is often induced by infection with cytoplasmatically inherited microorganisms, such as *Wolbachia*. *Wolbachia* are intracellular, symbiotic bacteria belonging to the order Rickettsiales within the α-Proteobacteria. *Wolbachia* is known to infect a wide range of arthropods, including insects, spiders, mites, scorpions and isopods, and has also been found in nematodes. A recent analysis estimated that 66% of all insect species is infected with *Wolbachia* (Hilgenboecker *et al.* 2008). *Wolbachia* is maternally inherited, because a sperm cell contains too little cytoplasm to harbour the bacteria. Therefore, they benefit from female-biased sex ratios in their hosts. To enhance its own transmission, *Wolbachia* can induce various alterations of the reproduction mechanism of its host, such as cytoplasmic incompatibility, feminization, male-killing and parthenogenesis. *Wolbachia*-induced parthenogenesis is most commonly found in haplodiploid organisms, such as Hymenoptera (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008).

Like all Hymenoptera, *T. coeruleus* is haplodiploid. Fertilized eggs develop into diploid daughters, while unfertilized eggs develop into haploid sons. This form of sexual reproduction is called arrhenotoky. Haplodiploid organisms can relatively easily shift to asexual reproduction. All that is required is diploidization of the haploid eggs, for example by alteration of meiotic and/or mitotic processes. It has been shown that *Wolbachia* can induce parthenogenesis in haplodiploid organisms by disrupting the segregation of the homologous chromosomes during the first mitotic division after meiosis in unfertilized eggs (Huigens & Stouthamer 2003, Pannebakker *et al.* 2004a). This form of parthenogenetic reproduction in haplodiploids is called thelytoky. Technically speaking, in sexually reproducing haplodiploids sons are also produced parthenogenetically, because they develop from unfertilized haploid eggs. In thelytokous reproducing haplodiploids, daughters are produced parthenogenetically from unfertilized diploid eggs. For convenience, in this paper we will use the terms sexual reproduction and parthenogenesis in stead of arrhenotoky and thelytoky.

Here, we investigate whether the ecological pattern found by Hoffmann *et al.* (2008) is also applicable within species. According to this pattern, parthenogenetic

populations of *T. coeruleus* should be found in agricultural environments, while sexual populations would be expected in natural environments. In support of this, Russell & Johnston (1912) and Johnston (1915) found parthenogenetic populations of *T. coeruleus* on the agricultural fields in northeastern United States. Therefore, we predicted that European agricultural populations of *T. coeruleus* would be similarly parthenogenetic. We further expected that natural populations of *T. coeruleus* might reproduce sexually. In addition, we investigated whether *Wolbachia* infection is the cause of parthenogenetic reproduction in *T. coeruleus*.

We sampled different populations of *T. coeruleus*. For each population, we determined field sex ratio and *Wolbachia* infection status. Parthenogenetic populations turned out to be infected with *Wolbachia*. Because we found differences in sex ratio and *Wolbachia* infection frequency between populations, we sequenced *Wolbachia* to see whether different populations were infected with different strains of *Wolbachia*.

Materials and Methods

Field sampling

In the spring and summer of 2007 and 2008, eleven populations of *Tetrastichus coeruleus* were sampled throughout The Netherlands and Belgium (Fig. 2.1). Several locations were visited in both years and multiple times per year. Five populations were sampled in coastal dune areas, five populations were sampled from agricultural fields and one population was sampled in a private allotment garden. Because the sampled agricultural field population from Antwerpen, Belgium, lies very close to the Dutch border and for convenience in this paper, we will talk about five Dutch agricultural fields. *T. coeruleus* was not found in dune areas north of the Noord-Hollandse Duinen and south of Goeree Overflakkee. In the summer of 2006 a population of *T. coeruleus* was sampled in a natural area in the Camargue in southern France (between Albaron and Saintes Maries de la Mer, on *Asparagus maritimus*) and in the spring of 2008 *T. coeruleus* was sampled on agricultural fields in Massachusetts, USA. In all locations, except the Camargue, adult wasps were caught in the field, giving the operational sex ratio of the population. In the Camargue and some locations in The Netherlands (Noord-Hollandse Duinen, Kennemerduinen, Meijendel, Gelderland and Brabant), larvae of *C. asparagi* were collected and reared in the lab. Some of these had been parasitized by *T. coeruleus*. Wasps emerging from the pupae of *C. asparagi* give the primary sex ratio of the population.

The sex of each wasp (caught in the field or emerged from pupae of *C. asparagi*) was determined by looking under a binocular for the presence of a groove on the ventral side of the abdomen which envelopes the ovipositor. In males this groove is absent. All wasps were then stored individually in 70% ethanol in a 1.5 ml Eppendorf tube and kept at -20°C.

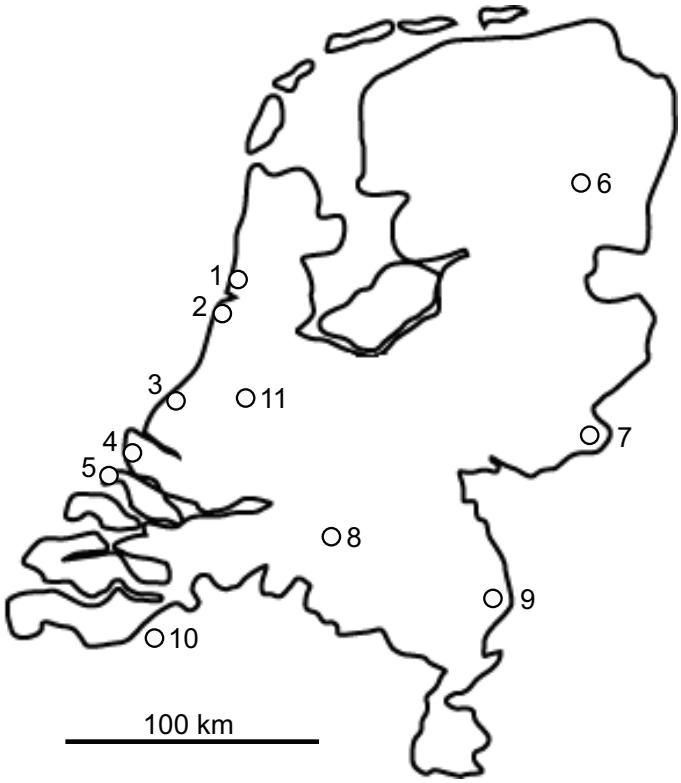

Figure 2.1: Map of collection sites of *Tetrastichus coeruleus* in The Netherlands. 1-5: Dune areas (1: Noord-Hollandse Duinen, 2: Kennemerduinen, 3: Meijendel, 4: Voornse Duinen, 5: Goeree Overflakkee), 6-10: Agricultural fields (6: Drenthe, 7: Gelderland, 8: Brabant, 9: Limburg, 10: Antwerpen), 11: Private allotment garden (Alphen aan den Rijn).

DNA extraction

DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, using mini spin columns. Before starting the DNA extraction, each wasp was transferred to a new 1.5 ml Eppendorf tube. After evaporation of remaining ethanol, tissue lysis buffer (ATL) was added to the tube and the wasp was crushed using a plastic pestle. The tissue was incubated overnight in proteinase K at 56°C. The DNA was dissolved in 100 µl elution buffer (AE).

Table 2.1: Primers used to amplify genes for *Wolbachia* detection and *Wolbachia* characterization (sequencing) in *Tetrastichus coeruleus*. For each gene the forward (F) and reverse (R) primer sequences with the specific annealing temperature T_a (°C) are given. Note that for the genes *wsp* and *ftsZ* different primers were used for sequencing than for *Wolbachia* detection.

Gene	Primer name	Primer sequence (5' → 3')	T_a	Reference
Wolbachia detection				
wsp	*wsp* 81F	TGG TCC AAT AAG TGA TGA AGA AAC	55	Braig *et al.* 1998
	wsp 691R	AAA AAT TAA ACG CTA CTC CA		Zhou *et al.* 1998
ftsZ	*ftsZ* F	GGA CCG GAT CCG TAT GCC GAT TGC AGA GCT TG	55	Holden *et al.* 1993
	ftsZ R	GGA CCG AAT TCG CCA TGA GTA TTC ACT TGG CT		Sinkins *et al.* 1995
Wolbachia characterization				
gatB	*gatB* Asp F1	TTT AGA GCA AGA TGC AGG RAA GAG CG	64	Baldo *et al.* 2006
	gatB R1	TGG YAA YTC RGG YAA AGA TGA		MLST website
gatB	*gatB* Bsp F1	TAA GAA TCG CAA GAA TTC AC	62	Baldo *et al.* 2006
	gatB R1	TGG YAA YTC RGG YAA AGA TGA		MLST website
coxA	*coxA* Asp F1	ATA CCC ACC TTT ATC ACA GG	56	Baldo *et al.* 2006
	coxA R1	CTA AAG ACT TTK ACR CCA GT		MLST website
coxA	*coxA* Bsp F1	ATA CCC ACC TYT RTC GCA AA	54	Baldo *et al.* 2006
	coxA R1	CTA AAG ACT TTK ACR CCA GT		MLST website
hcpA	*hcpA* F1	GAA ATA RCA GTT GCT GCA AA	55	Baldo *et al.* 2006
	hcpA Asp R1	TTC TAR YTC TTC AAC CAA TGC		MLST website
hcpA	*hcpA* F1	GAA ATA RCA GTT GCT GCA AA	55	Baldo *et al.* 2006
	hcpA Bsp R1	TTC TTT GTC GCT MAC TTY AAT CAK G		MLST website
ftsZ	*ftsZ* Asp F1	AAA GAT AGT CAT ATG CTT TTC	55	Baldo *et al.* 2006
	ftsZ Asp R1	CAT CGC TTT GCC CAT CTC G		MLST website
ftsZ	*ftsZ* Bsp F1	AAA GAT AGC CAT ATG CTC TTT	59	Baldo *et al.* 2006
	ftsZ Bsp R1	CAT TGC TTT ACC CAT CTC A		MLST website
fbpA	*fbpA* Asp F1	TTA ACC CTG ATG CTT ATG AC	55	Baldo *et al.* 2006
	fbpA R1	CCR CCA GAR AAA AYY ACT ATT C		MLST website
fbpA	*fbpA* Bsp F1	GTT AAC CCT GAT GCT TAC GAT	58	Baldo *et al.* 2006
	fbpA R1	CCR CCA GAR AAA AYY ACT ATT C		MLST website
wsp	*wsp* F1	GTC CAA TAR STG ATG ARG AAA C	59	Baldo *et al.* 2006
	wsp R1	CYG CAC CAA YAG YRC TRT AAA		MLST website

Wolbachia detection

Wasps were tested for *Wolbachia* infection by amplifying the *Wolbachia*-specific *wsp* gene, with forward primer *wsp*-81F and reverse primer *wsp*-691R (Braig *et al.* 1998, Zhou *et al.* 1998; for primer sequences and annealing temperatures see Table 2.1). When amplification of *wsp* was weak and infection status ambiguous, the *Wolbachia*-specific *ftsZ* gene was also amplified (Holden *et al.* 1993, Sinkins *et al.* 1995; for primer sequences and annealing temperatures see Table 2.1). Polymerase Chain Reactions (PCR) for both the *wsp* gene and the *ftsZ* gene were performed in a total volume of 20.0 µl, containing 1x PCR-buffer (Qiagen), 62.5 µM dNTPs, 1 unit Taq polymerase, 250 nM forward primer, 250 nM reverse primer and 1.0 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research, Waltham, MA, USA) was used for all PCRs. PCR conditions for the *wsp* gene were as follows: 3 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and finally 5 min at 72°C. PCR conditions for the *ftsZ* gene were as follows: 3 min at 94°C, then 35 cycles of 45 sec at 94°C, 1 min at 55°C and 1 min at 72°C and finally 5 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Wolbachia characterization

Wolbachia from three populations were characterized by sequencing six genes specific for *Wolbachia*. Two infected females were used from each population: the dune area of Meijendel in The Netherlands, the natural area in the Camargue in France and the agricultural fields in Massachusetts, USA. For each female, all six genes were sequenced. *Wolbachia* was characterized by sequencing a set of five Multi Locus Sequence Typing (MLST) genes: *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA* (Baldo *et al.* 2006; see also the MLST website: http://pubmlst.org/wolbachia/, Jolley *et al.* 2004). For these five genes, specific primers for both the A- and B-type *Wolbachia* alleles were tested. In addition to these MLST genes, the *Wolbachia*-specific *wsp* gene was sequenced (Baldo *et al.* 2006). For primer sequences and annealing temperatures see Table 2.1. Note that for the genes *wsp* and *ftsZ* different primers were used for sequencing than for *Wolbachia* detection. PCRs for both the MLST genes and the *wsp* gene were performed in a total volume of 40.0 µl, containing 1x PCR-buffer (Qiagen), 200 µM dNTPs, 0.5 unit Taq polymerase, 1.0 µM forward primer, 1.0 µM reverse primer and 2.0 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research) was used for all PCRs. PCR conditions for both the MLST genes and the *wsp* gene were as follows: 2 min at 94°C, then 37 cycles of 30 sec at 94°C, 45 sec at the specific annealing temperature and 90 sec at 72°C, and finally 10 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Sequencing was performed by Macrogen Inc. (Seoul, Korea). Sequences were checked with Sequencher software (version 4.2; Gene Codes, Ann Arbor, MI, USA)

and aligned with BioEdit software (version 7.0.9; Hall 2007). The resulting sequences were compared with each other and with other available sequences in the *Wolbachia* MLST profiles database on the MLST website (http://pubmlst.org/wolbachia/, Jolley *et al.* 2004), using several query functions on the website. The Allelic Profile Query was used to compare the combination of our five MLST alleles with other available allele combinations. The Similarity and Compare functions were used to compare the sequences of single alleles with other available alleles of the same gene in *Wolbachia*. For the *wsp* gene similar comparisons could be made using the *wsp* database on the MLST website.

Statistical analysis

Statistical analyses were performed in R (version 2.8.0; R Development Core Team 2008). Generalized linear models (glm) with a binomial error distribution were used to test for differences in sex ratio and infection frequency between populations. In the sex ratio model, the number of males was used as the response variable and the total number of individuals as the binomial denominator. Location was nested within habitat as an explanatory variable. Five habitats were included: Dutch dune areas (with five locations), Dutch agricultural fields (with five locations), the allotment garden, the natural area in France and the agricultural fields in the USA. To test for differences in collection method (wasps or larvae), only two habitats were considered (Dutch dunes and Dutch fields) and collection method was nested within habitat. In the infection frequency model, the number of infected females was used as the response variable and the total number of females as the binomial denominator (males were never infected). Location was nested within habitat as an explanatory variable. The same five habitats as in the sex ratio model were included. Significance of explanatory variables was tested by dropping terms from the model or lumping levels within terms and comparing the resulting change in deviance to a χ^2-distribution. This was done for each explanatory variable, until the simplest model remained, from which all non-significant terms and levels had been removed.

Results

Field sampling

The results of the field sampling are summarized in Table 2.2. At three sites we also collected *Tetrastichus crioceris*, a close relative of *T. coeruleus* that parasitizes the spotted asparagus beetle (*Crioceris duodecimpunctata*) that also lives on asparagus plants: three individuals from the Noord-Hollandse Duinen, ten from Meijendel and one from Antwerpen.

Table 2.2: Sex ratio and *Wolbachia* infection frequency for the field samples of *Tetrastichus coeruleus*. In almost all locations adult wasps were field-caught (column 2-9), while in some locations larvae of *C. asparagi* were collected and reared in the lab (column 10-14). Column 1 shows all locations. Column 2-9 give numbers (n) and percentages (%) of field-caught adult males and females, and infected and uninfected females. Column 10 indicates the number of pupae from the collected larvae that developed wasps. Column 11-14 give numbers (n) and percentages (%) of males and females that emerged from these pupae.

	Males		Females		Inf. females		Uninf. females		Pupae	Males		Females	
Location	n	%	n	%	n	%	n	%	n	n	%	n	%
Noord-Hollandse Duinen	0	0	12	100	11	91.67	1	8.33	2	0	0	14	100
Kennemerduinen	0	0	18	100	18	100	0	0	7	6	10.17	53	89.83
Meijendel	0	0	60	100	59	98.33	1	1.67	16	7	6.67	98	93.33
Voornse Duinen	1	3.85	25	96.15	24	96	1	4	-	-	-	-	-
Goeree Overflakkee	0	0	15	100	14	93.33	1	6.67	-	-	-	-	-
Total Dunes (NL)	1	0.76	130	99.24	126	96.92	4	3.08	25	13	7.3	165	92.7
Drenthe	6	7.69	72	92.31	0	0	72	100	-	-	-	-	-
Gelderland	8	7.34	101	92.66	0	0	101	100	63	219	45.82	259	54.18
Brabant	9	8.11	102	91.89	0	0	58	100	102	347	43.38	453	56.63
Limburg	6	17.65	28	82.35	0	0	28	100	-	-	-	-	-
Antwerpen	0	0	22	100	0	0	22	100	-	-	-	-	-
Total Fields (NL)	29	8.19	325	91.81	0	0	281	100	165	566	44.29	712	55.71
Alphen aan den Rijn (NL)	2	12.5	14	87.5	0	0	14	100	-	-	-	-	-
Camargue (FR)	-	-	-	-	69	100	0	0	16	0	0	69	100
Massachusetts (USA)	0	0	213	100	213	100	0	0	-	-	-	-	-

Sex ratio

In four of the five Dutch dune areas only females were caught. Only in the Voornse Duinen one male was collected (Table 2.2). On four of the five Dutch agricultural fields both males and females were found, although this operational sex ratio was highly female-biased on all four fields (Table 2.2). From the agricultural field in the province of Antwerpen only females were collected. Within the habitats Dutch dune areas and Dutch agricultural fields, there was no significant difference in sex ratio between locations (deviance = 10.29, $df = 8$, $p = 0.25$). Therefore, locations within these habitats were pooled for further analyses. In the Dutch allotment garden, both males and females were collected, with a highly female-biased sex ratio (Table 2.2). Both in the natural area in the Camargue (France) and on the agricultural fields in Massachusetts (USA) only females were found (Table 2.2). There was a significant difference in sex ratio between the five habitats (Dutch dunes, Dutch agricultural fields, the allotment garden, the Camargue and Massachusetts: deviance = 42.84, $df = 4$, $p < 0.0001$; Fig. 2.2). The sex ratios within locations appeared constant between seasons and years.

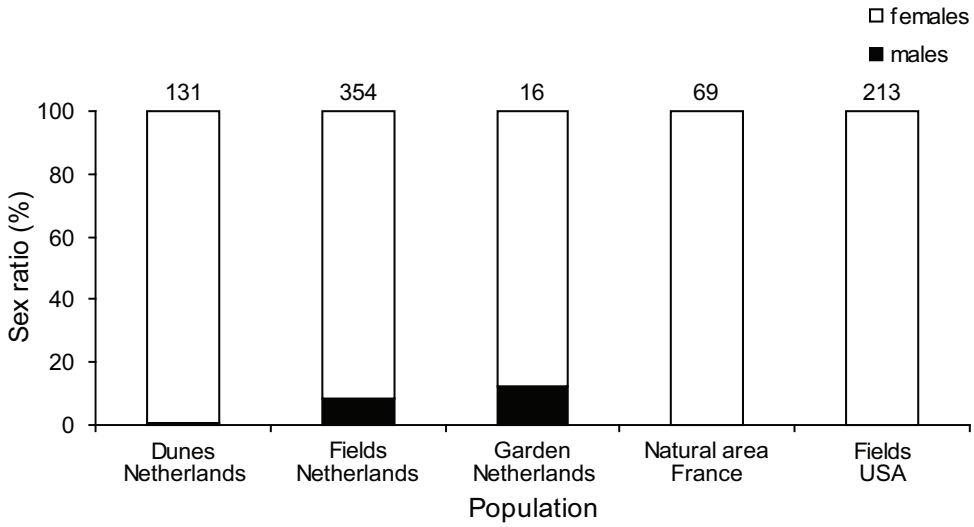

Figure 2.2: Sex ratio in five populations of *Tetrastichus coeruleus*: three field-caught populations from The Netherlands (one from five dune areas along the Dutch coast, one from five agricultural fields in the Dutch and Belgian inlands, and one from a private allotment garden), one lab-reared population from a natural area in the Camargue in southern France and one field-caught population from agricultural fields in Massachusetts (USA). Black areas represent percentage collected males and white areas represent percentage collected females. Sample sizes for each population are indicated above the bars.

The sex ratio in the Dutch dune population and the Dutch field population differed significantly from each other (deviance = 12.67, $df = 1$, $p < 0.001$). The sex ratio in the allotment garden did not differ significantly from that in the Dutch field population (deviance = 0.33, $df = 1$, $p = 0.57$), but did differ significantly from that in the Dutch dune population (deviance = 5.49, $df = 1$, $p = 0.02$). The sex ratios in France and the USA did not differ significantly from that in the Dutch dune population (France: deviance = 0.85, $df = 1$, $p = 0.36$; USA: deviance = 1.94, $df = 1$, $p = 0.16$), but differed significantly from that in the Dutch field population (France: deviance = 10.74, $df = 1$, $p = 0.001$; USA: deviance = 28.26, $df = 1$, $p < 0.0001$). The sex ratios between France and the USA did not differ significantly from each other (deviance < 0.0001, $df = 1$, $p = 0.99$).

When larvae of *C. asparagi* were collected and reared in the lab, a higher percentage of *T. coeruleus* males emerged from the pupae than when wasps were field caught. This was true for two dune areas (Kennemerduinen and Meijendel; Table 2.2 and for two agricultural fields (Gelderland and Brabant; Table 2.2). Within both habitats the sex ratios of lab-reared populations (the primary sex ratios) were significantly higher (more males) than the sex ratios of field-caught populations (the operational sex ratios) (deviance = 194.76, $df = 2$, $p < 0.0001$). Within the two habitat categories there was no significant difference in primary sex ratio between locations (deviance = 3.55, $df = 3$, $p = 0.31$), but there was a significant difference in primary sex ratio between the dune areas and the agricultural fields (deviance = 108.99, $df = 1$, $p < 0.0001$).

Wolbachia detection

Almost all females in the five Dutch dune areas were infected with *Wolbachia*. In each of four dune areas a single uninfected female was found (Table 2.2). In the Kennemerduinen only infected females were found. Unexpectedly, none of the females collected on the Dutch agricultural fields were infected with *Wolbachia* (Table 2.2). Within the habitats Dutch dune areas and Dutch agricultural fields, there was no significant difference in infection frequency between locations (deviance = 2.92, $df = 8$, $p = 0.94$). Therefore, locations within these habitats were pooled for further analyses. None of the females in the Dutch allotment garden were infected with *Wolbachia*, while both in the natural area in the Camargue (France) and on the agricultural fields in Massachusetts (USA) all females were infected (Table 2.2). There was a significant difference in infection frequency between the five habitats (Dutch dunes, Dutch agricultural fields, the allotment garden, the Camargue and Massachusetts; deviance = 927.51, $df = 4$, $p < 0.0001$; Fig. 2.3).

The infection frequencies in the Dutch dune population and the Dutch field population differed significantly from each other (deviance = 470.90, $df = 1$, $p < 0.0001$). The infection frequency in the allotment garden did not differ significantly from that in the Dutch field population (deviance < 0.0001, $df = 1$,

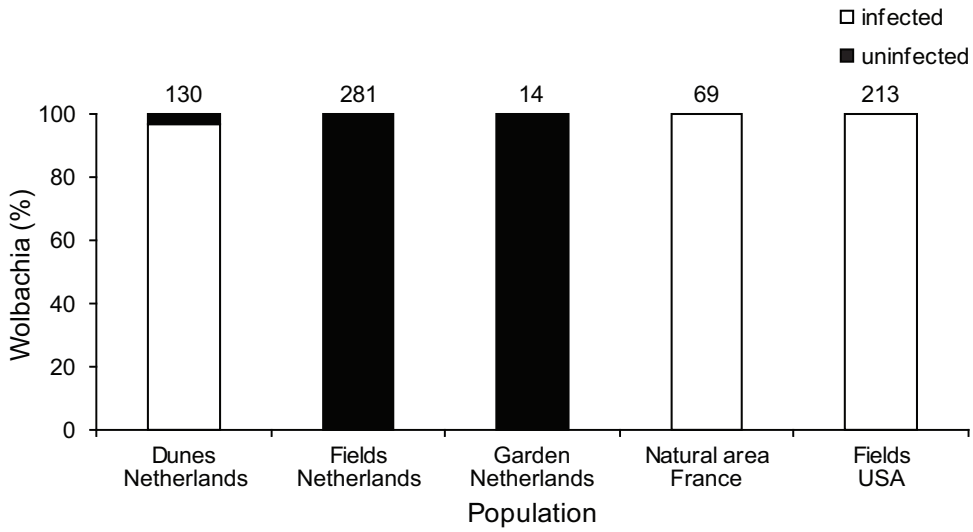

Figure 2.3: *Wolbachia* infection frequency of females in five populations of *Tetrastichus coeruleus*: three field-caught populations from The Netherlands (one from five dune areas along the Dutch coast, one from five agricultural fields in the Dutch and Belgian inlands, and one from a private allotment garden), one lab-reared population from a natural area in the Camargue in southern France and one field-caught population from agricultural fields in Massachusetts (USA). White areas represent percentage *Wolbachia*-infected females and black areas represent percentage uninfected females. Sample sizes for each population are indicated above the bars.

$p = 1$), but was significantly different from that in the Dutch dune population (deviance = 72.78, $df = 1$, $p < 0.0001$). The infection frequencies in France and the USA did not differ significantly from each other (deviance < 0.0001, $df = 1$, $p = 1$). The infection frequency in the USA differed significantly from that in the Dutch dune population (deviance = 7.84, $df = 1$, $p = 0.005$). However, the infection frequency in France did not differ significantly from that in the Dutch dune population (deviance = 3.45, $df = 1$, $p = 0.06$). This is probably due to the smaller sample size from the Camargue compared to that from the dune population. Both infection frequencies in France and the USA differed significantly from that in the Dutch field population (France: deviance = 4974.0, $df = 1$, $p < 0.001$; USA: deviance = 15354.6, $df = 1$, $p < 0.001$).

Wolbachia characterization

Wolbachia from three infected populations (Dutch dunes, natural area in France and agricultural fields in USA) were characterized by sequencing six *Wolbachia*-specific genes. For the five MLST genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) all B-type *Wolbachia*-specific alleles were amplified, while for *fbpA* also the A-type specific allele was amplified. However, both sequences for the *fbpA* gene were exactly the same. It can thus be concluded that *T. coeruleus* is infected with *Wolbachia* belonging to *Wolbachia*-supergroup B and is infected with only one *Wolbachia* strain. *Wolbachia* from the three different populations of *T. coeruleus* were genetically identical (100% similarity). This *Wolbachia* strain, which we name *wTcoer* has sequence type (ST) 37 with alleles *gatB*-9, *coxA*-9, *hcpA*-6, *ftsZ*-8 and *fbpA*-10, and *wsp*-63, with highly variable regions HVR1-19, HVR2-17, HVR3-24 and HVR4-33. The sequences belonging to *wTcoer* have been submitted to the GenBank database (accession numbers GU724211-GU724216) and to the MLST website (id 293; http://pubmlst.org/wolbachia/).

Discussion

We investigated whether the pattern found by Hoffmann *et al.* (2008) also is applicable within the species *Tetrastichus coeruleus*. According to this pattern, parthenogenetic populations should be found in agricultural environments, while sexual populations would be expected in natural environments.

The results showed that there are two main populations of *T. coeruleus* in The Netherlands. One population was found on wild asparagus plants in the dune areas. This population consisted almost exclusively of females (less than 1% males in the field and 7% males emerging from pupae) and was infected with *Wolbachia*. The populations from the five different dune areas did not differ in sex ratio or *Wolbachia* infection status. Previous work in our lab showed that females from the dune population reproduce through parthenogenesis, but that their offspring is not 100% female (B. Wielaard, pers. comm.). The other population is found on cultivated asparagus plants in agricultural fields. This population contained both males and females, although with a strongly female-biased operational sex ratio in field-caught adults (8% males). However, the primary sex ratio among the emerging wasps from *C. asparagi* larvae that were collected in the field and reared in the lab was much higher (44% males). An explanation for this difference between operational and primary sex ratio might be that males have a smaller probability of being found on the asparagus plants and are therefore less easily caught, or that females live longer than males. None of the wasps sampled from this population were infected with *Wolbachia*. Again, we found no differences between the five populations from the agricultural fields in terms of sex ratio or *Wolbachia* infection status. The population in the allotment garden resembled those in the Dutch agricultural fields: both males and females were found and none of them were infected with *Wolbachia*.

This population has probably been transported from the agricultural fields to this garden, most likely as eggs or pupae in the root stems of asparagus plants. Both the French natural population and the American agricultural field population consisted entirely of females, which were all infected with *Wolbachia*. Given the perfect match between *Wolbachia* infection status and sex ratio, we suggest that *Wolbachia* causes *T. coeruleus* to reproduce through parthenogenesis (Russell & Johnston 1912, Johnston 1915, B. Wielaard, pers. comm.).

In a recent study, Hoffmann *et al.* (2008) found a high incidence of parthenogenesis in agricultural pest species. They found that in North America and Italy 45-48% of insect agricultural pest species reproduce through parthenogenesis compared to an overall incidence of only 10-16% parthenogenesis in these genera. This difference is thought to be due to the agricultural environments being simplified, uniform and rich in resources, such that the same genotype may be continuously favoured over other genotypes. Also, agricultural fields generally are regularly cleared of all vegetation, offering an advantage to parthenogenetically reproducing species due to their greater colonization ability (Glesener & Tilman 1978, Maynard Smith 1978, Haag & Ebert 2004). The Dutch *T. coeruleus* populations show the opposite of Hoffmann *et al.*'s (2008) pattern within one species: the agricultural field population reproduced sexually, whereas the natural dune population reproduced parthenogenetically. In the French natural population, only infected females were found, similar to the Dutch natural dune population. The similarity between the Dutch and French populations suggests that the presence or absence of *Wolbachia* is due to ecological selection on either the mode of reproduction or *Wolbachia* infection. At this moment, we do not have a convincing explanation for this deviation from the general pattern of ecology and reproductive mode. It is possible that the culture of asparagus plants differs from that of other crops in ways that offer an advantage to sexual reproduction. Asparagus fields are typically used for 10 or 15 years without crop rotation, whereas other agricultural fields have a very high turn over rate of crop species. Alternatively, there may be important differences between phytophagous pest species and their natural enemies. Hoffmann *et al.* (2008) focused on phytophagous insect pests, while we studied a parasitoid. It is also possible that *Wolbachia* manipulates life history trade-offs in its host in ways that are beneficial in natural, but not in agricultural environments. *Wolbachia* has been shown to affect life history traits in several of its hosts (Alexandrov *et al.* 2007, Brownlie *et al.* 2009, Gross *et al.* 2009, Jia *et al.* 2009).

Our findings in the American agricultural fields, however, were inconsistent with the European pattern: we found exclusively females, which reproduce through parthenogenesis (Russell & Johnston 1912, Johnston 1915). The American population originates from a (presumably small) founder population that was introduced from Europe. The beetles and wasps were most likely transported as eggs or pupae in the root stems of asparagus plants. Possibly, all of the introduced individuals

of *T. coeruleus* were infected with *Wolbachia*, giving rise to a founding population that was fixed for *Wolbachia*. Although these parthenogenetic populations may have a disadvantage in the agricultural fields, no uninfected, sexual source populations were available to invade and outcompete the parthenogenetic populations. As a consequence, all of the populations of *T. coeruleus* that arose from this founding population are completely infected with *Wolbachia* and reproduce parthenogenetically. It seems likely that the asparagus plants that were introduced into America originated from agricultural fields in Europe. This suggests that *Wolbachia* used to be present in *T. coeruleus* populations on European agricultural fields, and now has disappeared. Alternatively, there may be *Wolbachia*-infected *T. coeruleus* populations on European agricultural fields that we do not know of.

Only a few other parasitoid wasp species are known in which both sexual and parthenogenetic populations occur. In the parasitoid wasps *Apoanagyrus diversicornis* (Pijls *et al.* 1996), *Telenomus nawai* (Arakaki *et al.* 2000), *Leptopilina clavipes* (Pannebakker *et al.* 2004b) and *Asobara japonica* (Kremer *et al.* 2009) infected and uninfected populations occur allopatrically. Mixed populations of infected and uninfected individuals are limited to a number of species of the genus *Trichogramma* (Stouthamer *et al.* 1990a, 1990b, 2001). In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the whole population reproduces parthenogenetically (Huigens & Stouthamer 2003). In the sampled French and American populations of *T. coeruleus* the *Wolbachia* infection is indeed fixed. However, the *Wolbachia* infection is not fixed in the Dutch dune population. We found several males and uninfected females (Table 2.2). The origin of these individuals is currently unclear.

A reason for the difference between the Dutch dune population and the French and American populations could have been that they are infected with different strains of *Wolbachia* that have different effects on their hosts. However, all three populations harbour exactly the same *Wolbachia* strain. There were no differences in the sequences of the five *Wolbachia*-specific MLST genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) or the *wsp* gene.

Another explanation for the few uninfected females and males in the dune population might be inefficient transmission of the *Wolbachia* bacteria from mother to daughter. Infected females may loose their *Wolbachia* when they become older or because of high temperatures (Cabello & Vargas 1985, Legner 1985). Eggs laid in a later stage of life or at high temperatures would then be deprived of *Wolbachia* bacteria. The temperature in the dunes can become very high in certain areas. However, in the French and American populations of *T. coeruleus* we only found females that are all infected with *Wolbachia*, suggesting that there is a very efficient transmission of *Wolbachia* in these populations, even though the summers in southern France and Massachusetts can be very hot.

In *Trichogramma*, *Wolbachia* is prevented from reaching fixation by the action

of a supernumerary B chromosome, called Paternal-Sex-Ratio (PSR) chromosome. This is a selfish genetic element that is only transmitted through males and its evolutionary interests are thus opposed to those of maternally-inherited *Wolbachia* (Werren 1991, Beukeboom & Werren 2000, Stouthamer *et al.* 2001, Werren & Stouthamer 2003). After fertilization of an egg with the sperm of a PSR-infected male, the whole paternal genome, except for PSR, forms a dense chromatin mass and is eventually lost (van Vugt *et al.* 2003). This results in a haploid egg, which develops into a male with the extra PSR chromosome (Werren 1991, Werren & Stouthamer 2003). Since the PSR chromosome only acts after fertilization of the egg and *Wolbachia* is already transmitted to the egg via the cytoplasm during egg formation, the males that would develop from these eggs should be infected with *Wolbachia* (Stouthamer *et al.* 2001). However, none of the *T. coeruleus* males were infected with *Wolbachia*. Also, the presence of a PSR chromosome in *T. coeruleus* would not explain the uninfected females in the dunes. We think it is unlikely that we will find a PSR chromosome in *T. coeruleus*.

We also discounted the possibility that *Wolbachia* in *T. coeruleus* from the Dutch dunes is infected with a bacteriophage (Gavotte *et al.* 2004) that kills the bacterium or diminishes the effect on its host *T. coeruleus*. No bacteriophage infections were found in *Wolbachia* in *T. coeruleus* (data not shown).

Perhaps the simplest explanation why *Wolbachia* is not fixed in the Dutch dunes is that the males and uninfected females we found migrated from the agricultural fields to the dune areas. If so, then migration appears to be unidirectional. No infected females were found on the agricultural fields.

Since only the B-type *Wolbachia*-specific alleles were amplified, we conclude that *T. coeruleus* is infected with a single *Wolbachia* strain belonging to *Wolbachia*-supergroup B. We name this strain *wTcoer*. Interestingly, the molecular profile of this *Wolbachia* was an exact match to that of the *Wolbachia* strain found in *Anthene emolus*, a lycaenid butterfly species from Malaysia (Russell *et al.* 2009). The presence of the same or very similar *Wolbachia* strains in very distantly related species is still poorly understood. In addition to vertical transmission, horizontal transmission seems to play an important role in the dispersal of *Wolbachia* (e.g. Vavre *et al.* 1999, Huigens *et al.* 2000, Noda *et al.* 2001, Kittayapong *et al.* 2003, Sintupachee *et al.* 2006, Raychoudhury *et al.* 2009).

In conclusion, we found that some populations of *T. coeruleus* are infected with a parthenogenesis-inducing *Wolbachia*. Infection status and mode of reproduction of European populations follow an ecological pattern, with infected, parthenogenetic populations being limited to natural areas and uninfected, sexual populations limited to agricultural fields. This is remarkable, since agricultural fields are generally thought to be simplified and favour asexual reproduction, while natural environments are considered to be complex and favour sexual reproduction.

Acknowledgements

We want to thank all the Dutch and American farmers for allowing us to collect wasps on their asparagus fields and the Duinwaterbedrijf Zuid-Holland for granting us access to the restricted areas of the Meijendel dunes. We also want to thank Bas Wielaard for his information on parthenogenesis in *T. coeruleus* and Martine Maan for her insightful comments on the manuscript. This research was supported by a grant from the Netherlands Organization for Scientific Research, division Earth and Life Sciences (NWO-ALW), ref. no. 814.01.009, awarded to JJMvA.