Infection of Bois-Noir tuf-type-I stolbur phytoplasma in *Hyalesthes obsoletus* (Hemiptera: Cixiidae) larvae and influence on larval size

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Abstract

Recent dramatic spread of the grapevine yellows disease Bois Noir (BN) in Germany is above all explained by highly increased abundances of the vector *Hyalesthes obsoletus* (Hemiptera: Cixiidae) associated to the plant *Urtica dioica*, the reservoir of the BN pathogen stolbur tuf-type-I. The vector acquires BN-phytoplasma as larvae whilst feeding on the roots of infected *U. dioica*. To understand the dynamics of the *Urtica*-cycle, we tested at what instar larvae become infected and whether infection affects larvae size (i.e. growth) at two sites in the Mosel Valley, Germany. Larvae were tested from infected plants and collected at instar-stages 3, 4 and 5. Larvae at stage 3 were already infected but infection rates increased significantly between stage 3 and 5, mean infection rates: 0.12–0.62. There was no effect of infection on larval size at any instar stage.

Introduction

Vector-borne pathogens belong to some of the most economically important disease-causing organisms in agricultural systems. Plant-pathogen-vector dynamics may be very complex due to both direct and indirect interactions between the organisms (Belliure et al. 2005). Key factors for dissemination are survival of the vector and its ability to transmit the pathogen. Pathogens depend on the herbivore vector for transmission and dispersal but may also affect the herbivore negatively because of their presence (Bressan et al. 2005a,b). Furthermore, the pathogen and the herbivore vector can both induce responses in the host plant, thereby affecting each other indirectly through the plant (Bressan et al. 2005c; Mayer et al. 2008). Elliot et al. (2003) showed that in a patchy environment parasites should become benign towards the vector when it is the more mobile species, free parasites rarely disperse and parasite strains do not compete. However, virulence may increase if vectors feed on new hosts with novel 'maladapted' strains or by competition between different pathogen strains (Elliot et al. 2003). Fitness benefits of infection to the vector include increased longevity and fecundity (Beanland et al. 2000) and survival (Ebbert and Nault 2001; Belliure et al. 2005), whilst studies showing negative effects include reduced life span of the vector *Paraphlesius irroratus* (Say) infected with Eastern-X disease phytoplasma (Garcia-Salazer et al. 1991) and lower fecundity and longevity of *Scaphoideus titanus* Ball infected by flavescence dorée phytoplasma (Bressan et al. 2005b).

The planthopper *Hyalesthes obsoletus* Sign. is the vector of the grapevine yellows disease Bois Noir (BN) (Maixner 1994; Maixner et al. 1995; Sforza et al. 1998). Bois Noir is caused by stolbur (16SrXII-A) phytoplasmas (Maixner et al. 1995), which are transmitted to new plants via feeding adult vectors. Transmission to grapevine is an accidental outcome of the vector's polyphagous foraging behaviour, as

the vector neither reproduces on nor can acquire the phytoplasma pathogens from grapevines. For the phytoplasma, grapevine is a dead-end host (Boudon-Padieu 2000). The main host- and reservoir plants of *H. obsoletus* and stolbur in European vineyards are field bindweed (*Convolvulus arvensis* L.) and stinging nettle (*Urtica dioica* L.). The two host plants harbour specific stolbur strains, BN tuf-type II and BN tuf-type I respectively (Langer and Maixner 2004), which are also found in *H. obsoletus* caught on the respective plants. Nymphs of *H. obsoletus* acquire phytoplasmas whilst feeding on the roots of the host plants. Hence, adult planthoppers are infectious upon emergence. Different flight periods of *H. obsoletus* emerging from the alternative plants (Maixner et al. 2006) and slight but significant genetically differentiated host-plant populations (Johannesen et al. 2008) at a syntopic site in Germany suggest the existence of different disease cycles.

Whilst the bindweed cycle (*C. arvensis–H. obsoletus–Vitis vinifera*) until recently was the predominant cycle in Germany and in other central European countries (Suchov and Vovk 1948; Blattný et al. 1954; Brčak 1955; Sforza et al. 1999), the nettle cycle (*U. dioica–H. obsoletus–V. vinifera*) has become the most important in Germany within the last 10 years. This new disease cycle, which is also predominant in Italy (Alma et al. 1987; Lessio et al. 2007), is the primary reason for the dramatic increase of BN in Germany (Maixner 2006). The increase of BN is associated to very high abundances of *H. obsoletus* on *U. dioica* with infection rates of up to 30–40% in *H. obsoletus* (Bressan et al. 2007; Lessio et al. 2007; Maixner et al. 2007). The infection rates and the proliferation of *H. obsoletus* on *U. dioica* may suggest positive effects of stolbur infestation to the vector.

In the present study we report on stolbur infestation in *H. obsoletus* larvae feeding on *U. dioica*. We analyse the progress of infection during spring and whether infection influences larval size. Because the time to metamorphosis of imagos is a function of larval growth that occurs as discrete instar steps, larval size may be linked to fitness by increasing survival and by allowing early maturation and a prolonged fertility period (Danks 2007).

Material and Methods

Collections

Larvae from the third to fifth instars of *H. obsoletus* were analysed from stolbur infected *U. dioica* collected in two vineyards in the Mosel Valley, Germany, Lehmen (50°16′N) and Koblenz–Güls (50°20′N). Larvae were collected on roots of *U. dioica* after gently removing the topsoil. Roots with larvae were cut off and brought to laboratory for tests of stolbur infestation. All larvae were associated to individual roots (plants). Sampling of larvae and roots was done in 2007 at five dates in both Lehmen (15.03.2007–18.06.2007) and Güls (25.03.2007–25.05.2007). Larvae were analysed from 16 stolbur positive plants (Lehmen 9, Güls 7). Larvae were stored in ethanol (70%) until analysis.

DNA analysis

Total genomic DNA of *H. obsoletus* was extracted from each individual using the Roche Diagnostics 'High Pure PCR Template Preparation Kit' (Roche Diagnostics, Mannheim, Germany). Extraction of *U. dioica* genomic DNA followed the method of Wang et al. (1993). A total of 10 mg root tissue was ground in a Retch mill (Retch MM 301, Hann, Germany) for 3 min at 30 Hz. The plant tissue was added 400 μl 0.5 M NaOH and centrifuged for 10 s at 16060 ***g*** (13000 rpm HERAEUS Biofuge Pico). Finally, 5 μl of the supernatant with DNA was diluted with 95 μl 0.1 M Tris-buffer (pH 8.0) and stored at −20°C until use.

Stolbur infection in *U. dioica* and *H. obsoletus* was assessed with the stolbur specific primers STOL-11f2: 5′-TAT-TTT-CCT-AAA-ATT-GAT-TGG-C-3′ and STOL-11r1: 5′-TGT-TTT-TGC-ACC-GTT-AAA-GC-3′ (Daire et al. 1997). The amplification was performed in an end volume of 25 μl consisting of 1 μl forward and backward primer (10 pM/μl), 3 μl DNA extract and 20 μl H_2O sterile. Each sample was covered with 15 μl Chill Out 14 Liquid Wax (MJ Research, Watertown, MA, USA). The polymerase chain reaction (PCR) was performed with 'Ready ToGo™ PCR Beads' (0.5 ml tubes; Amersham Pharmacia Biotech, Nürnbrecht, Germany) using a PTC-100 thermocycler (MJ Research). The PCR reaction was started by denaturing at 94°C for 240 s, followed by 34 cycles: denaturation (30 s at 94°C), annealing (60 s at 55°C) and elongation (45 s at 72°C). The PCR was terminated with a final extension at 72°C for 30 s.

Polymerase chain reaction products were electrophoresed on 1.02 % agarose gel at 160 V for 1 h, visualized with ethidium bromide and photographed digitally (Canon Power Shot G5, Canon Deutschland, Krefeld, Germany) under UV light (UV transilluminator; Bioblock Scientific, Frenkenburg, Switzerland). Fragment lengths were analysed with the program BioDoc Analyse (Whatman Biometra,

Göttingen, Germany) by laying reference lines estimated from a 100 bp DNA ladder (Invitrogen). Amplification of the correct PCR product was confirmed by sequencing *U. dioica* and *H. obsoletus* amplifications (results not shown).

Measurement of *Hyalesthes obsoletus*

Larval stage was determined using the guidelines by Sforza et al. (1999). To test the influence of infection on larval size, we measured body length as the length from the tip of the head to the end of abdomen, and body width was the maximum width of thorax. Measurements were done under a Wild photomicroscope M400 (Wild, Leitz, Vienna, Austria) and photographed with a digital camera (Panasonic GP-KR 222, Panasonic Europe, Wiesbaden, Germany). Measurements were analysed with ImageTool (developed at the University of Texas Health Science Center at San Antonio, Texas; available by anonymous FTP from maxrad6.uthscsa.edu). All measurements were standardized with 1 mm plotting glass.

Statistical analysis

Correlations between body length and width were tested with Pearson coefficients. Infection rates amongst sampling dates and amongst instar classes were analysed with Chi-square tests with the program StatTools (http://department.obg.cuhk.edu.hk/researchsupport/statmenu.asp). To test the influence of infection on larval size we used two-way tests of ANOVA with the categorical variables: site (Lehmen, Güls) and infestation (no, yes). Homoscedasity was tested with Bartletts test. Two-way ANOVAS were performed separately for each instar class (L3, L4, L5) as these differed significantly in size (see results). In separate one-way ANOVAS we tested effects of plant and sampling date on larval size. Due to lack of homoscedasity amongst instar classes, size difference amongst instars was tested with the non-parametric Kruskal–Wallis test. ANOVAS and Kruskal–Wallis tests were performed with JMP (SAS Institute, 1995).

Results

Infection rates

In total, 286 *H. obsoletus* larvae (Lehmen 161, Güls 125) were analysed for size differences from 16 stolbur positive *U. dioica* plants (Lehmen 9, Güls 7). The larvae included 173 third (L3), 29 fourth (L4) and 84 fifth (L5) instar larvae. Amongst instar classes, 21 (12.1 %) L3, 9 (31 %) L4 and 52 (61.9 %) L5 larvae tested stolbur positive.

Tests for differing infection rates at different dates could be performed for L3 instars at Lehmen (15.03, 02.04 and 23.04) and Güls (25.03, 27.04 and 20.04) for each three sampling dates, and for L5 larvae at Güls between two sampling dates (14.05 and 25.05). Numbers of infected L4 and L5 larvae at Lehmen and L4 larvae at Güls on consecutive sampling dates were too low for differentiation tests. Infection rates within instars did not differ at different dates (L3: Lehmen, $\chi^2 = 3.54$, d.f. = 2, P = 0.17; L3: Güls, $\chi^2 = 1.52$, d.f. = 2, P = 0.47; L5: Güls, $\chi^2 = 0.67$, d.f. = 1, P = 0.41). However, the mean infection rate increased from L3 to L5 at both sites (Lehmen: L3 = 0.08, L4 = 0.43, L5 = 0.75, $\chi^2 = 69.60$, d.f. = 2, $P < 0.001$; Güls: L3 = 0.21, L4 = 0.27, L5 = 0.52, $\chi^2 = 11.00$, d.f. = 2, $P < 0.01$).

Infection and size

Within instar classes, measurements of length and width were highly correlated, Pearson correlation coefficients, r = 0.67–0.76, all $P < 0.001$. We therefore restricted the size analyses to length differences.

Instars classes differed significantly in size, $\chi^2 = 212.9$, d.f. = 2, $P < 0.001$ (mean ± standard deviation: L3 = 1.56 mm ± 0.22, L4 = 2.56 mm ± 0.29, L5 = 3.44 mm ± 0.50. Size variance within instars increased with increasing larval stage. Thus, variance amongst classes was not homogeneous (Bartletts test, F = 40.41, $P < 0.0001$).

There were no effects of stolbur infestation on size in any instar-classes (fig. 1, table 1). In L3 and L4 larvae there was a site effect, $P < 0.05$, and in L4 significant interaction between site and infection,

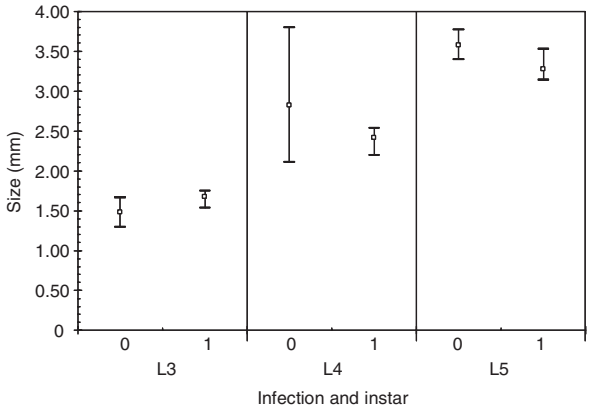

Fig. 1 Length (mm) of *Hyalesthes obsoletus* Sign. larvae at stage of development (L3, L4, L5) and state of infection (0 = not infected, 1 = infected) (□ = median and 95 % confidence interval).

Table 1 Two-way ANOVAS for the effects of site (Lehmen, Güls) and stolbur infection (yes, no) on the length of *H. obsoletus* larvae. Mean lengths of infected and non-infected larvae of each size-class are presented in the results and shown in fig. 1

Instar	Source	SS	d.f.	F-ratio	P
L3	Site	0.403	1	8.778	0.004
	Infection	0.016	1	0.349	0.556
	Site *x* infection	0.001	1	0.012	0.912
L4	Site	0.470	1	6.834	0.015
	Infection	0.039	1	0.566	0.459
	Site *x* infection	0.385	1	5.596	0.026
L5	Site	0.109	1	0.437	0.51
	Infection	0.134	1	0.537	0.46
	Site *x* infection	0.499	1	0.161	0.16

P = 0.026. No site and interaction effects were observed in L5 larvae. *Post-hoc* ANOVA analyses examining the effects of date and plant on size separately showed that the significant site effect on L3 larvae was caused by a skewed sample including many individuals from one plant in Lehmen; plant effect: $F_{7,165} = 9.162$, $P < 0.0001$. In L4 and L5 larvae there were no plant or date effects on larval size, L4 plant: $F_{4,24} = 1.198$, $P = 0.33$; L4 date: $F_{2,26} = 1.87$, $P = 0.17$; L5 plant: $F_{5,78} = 2.06$ $P = 0.08$; L5 Date: $F_{3,80} = 1.27$, $P = 0.29$).

Discussion

This is the first report of temporal acquisition of stolbur tuf-type I phytoplasma by *H. obsoletus* larvae on the host plant *U. dioica* and of potential effects of stolbur infestation in the larval stage. Stolbur detection has previously been shown in field collected L5-*H. obsoletus* from bindweed (Sforza et al. 1998). Size of larvae was used as a fitness component as adult emergence depends on larval growth that occurs as discrete instar steps and critical weight for metamorphosis (e.g. D'Amico et al. 2001; Etile and Despland 2008). Early metamorphosis may result in higher mating probability and/or prolonged reproductive period (Danks 2007). In the present work, PCR detection of phytoplasma revealed that the within-instar rate of infection did not change relative to the sampling date whereas infection rates increased significantly between instars L3 and L5. This result implies that infection does not affect maturation of instars and that trans-ovarial transmission is not of general importance for stolbur phytoplasma acquisition in *H. obsoletus*. Increased infection rates in L5 can be caused either by a higher probability for stolbur infection as a function of time or by higher survival of infected larvae. Only the latter will be of direct fitness benefit. It should also be noted that higher rate of infection in L5 than in L3 could be caused by methodical bias if low levels of phytoplasma in small L3 cannot yet be detected, e.g. due to recent acquisition. Phytoplasma titres are strongly increasing during latency (Boudon-Padieu et al. 1989). This would indicate progressive and increasing titre during larval development. From the data we cannot discriminate between the options but the lack of size effects amongst infected and non-infected *H. obsoletus* in all larval stages may suggest the former; i.e. there were no apparent fitness effects of infection in *H. obsoletus* larvae on *U. dioica*.

Despite the importance of vector-pathogen interactions in the dissemination of vector-borne plant diseases, data on vector fitness is scarce. Both positive (Beanland et al. 2000; Ebbert and Nault 2001; Belliure et al. 2005) and negative (Garcia-Salazer et al. 1991; Bressan et al. 2005b) effects to infected vectors have been reported. The data compiled in our study point against size effects caused by infection in the larval stage, which could indicate evolution towards less virulence in an essential host plant. If the *U. dioica* infectious cycle is caused by plant-specific *H. obsoletus* (i.e. host races) then the interaction between BN-phytoplasma and *H. obsoletus* may be expected to be positive or neutral. If the interaction is neutral, the recent increase of abundance of *H. obsoletus* on *U. dioica* may simply reflect changing abiotic environments favouring survival of the vector. However, preliminary experiments for longevity in adult *H. obsoletus* indicated that infected adults live longer (M. Maixner, unpublished data). In the well studied pea aphid *Acyrthosiphon pisum* (Harris), fitness benefits by secondary symbionts include e.g. parasitoid resistance (Oliver et al. 2003) and tolerance to heat stress (Chen et al. 2000). An indirect positive effect of infection was found to help adaptation to alternative host plants (Tsuchida et al. 2004). The latter two findings are of great interest in the study of *H. obsoletus* and the spread of BN, which in Germany is related to increased summer temperatures and specific host-plant disease cycles. In addition to our study of phytoplasma infection of *H. obsoletus* larvae on *U. dioica*, studies of larval infection on the reservoir plant *C. arvensis* are needed.

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