

1 **A genetic analysis reveals low prevalence of phytoplasma infection in *Hyalesthes***  
2 ***obsoletus* Signoret, vector of 'bois noir', in SW-Germany**

3

4 **Gabriele Schiro<sup>1</sup>, Johannes Fahretrapp<sup>2</sup>, Florian Hartig<sup>1</sup> and Bernd Panassiti<sup>3</sup>,**

5

6 1) University of Freiburg, Department of Biometry and Environmental System  
7 Analysis, Tennenbacherstraße 4, 79106 Freiburg, Germany

8 2) Research Group for Viticulture, Zurich University of Applied Sciences ZHAW,  
9 Department of Life Sciences and Facility Management, Grüental, CH-8820  
10 Wädenswil, Switzerland.

11 3) State Institute of Viticulture and Oenology, Merzhauser straÙe 119, 79100,  
12 Freiburg, Germany; present address: Laimburg Research Centre for Agriculture and  
13 Forestry

14 Laimburg 6 – Pfatten/Vadena, 39040 Auer/Ora (BZ), Italy.

15

16

17 **For Correspondence please contact:**

18 Bernd Panassiti, Laimburg Research Centre for Agriculture and Forestry, Laimburg 6  
19 Pfatten/Vadena, 39040, Auer/Ora, (BZ) Italy

20 Fax: +39 0471 969 555

21 Tel.: +39 0471 969 669

22 email: [bernd.panassiti@provinz.bz.it](mailto:bernd.panassiti@provinz.bz.it)

23

24

25

26 **Abstract**

27 Bois Noir is a grapevine disease responsible for severe economic losses in wine  
28 production. Bois Noir is caused by *Candidatus* Phytoplasma solani, cell wall-less  
29 bacteria belonging to the taxonomic group 16SrXII-A. In Germany, they are known to  
30 be vectored from plant to plant by the cixiid *Hyalesthes obsoletus* (Signoret), but the  
31 prevalence of the disease in the vector population, as well as its spatio-temporal  
32 distribution is poorly understood. Here, we analyzed infections of *H. obsoletus*  
33 individuals collected in different vineyards in Baden (South-Western Germany) with  
34 quantitative real-time PCR. From the 125 collected analyzed individuals, only five  
35 were infected with *Ca. P. solani*. All infected individuals were colonized by *Ca. P.*  
36 *solani* tuf-type a, which is associated with the host plant *Urtica dioica* (stinging  
37 nettle). We conclude that more research is needed to understand the reasons of this  
38 surprisingly low prevalence of Bois Noir infections in the population of *H. obsoletus*  
39 in South-West Germany.

40 **Key Words**

41 body size, Bois noir, *Hyalesthes obsoletus*, infectious disease, Phytoplasma,  
42 planthopper, *Vitis Vinifera*

44 **Introduction**

45 Phytoplasma are cell-wall less prokaryotes. Lacking common metabolic pathways,  
46 they need host tissues for their survival and are therefore obligate parasites  
47 (Christensen *et al.*, 2005). Phytoplasma have developed complex life cycles that  
48 involve replications in both insects and plants. In plants, they are usually found in  
49 phloem tissues, while in insects they need to cross the gut cells, replicate within  
50 internal tissues, and then reach salivary glands for transmission to new plants  
51 (Hogenhout *et al.*, 2008). This peculiar life cycle allows them to easily reach new host  
52 plants taking advantage of the mobility provided by the insect hosts. In cultivated  
53 crops, they can also be transmitted through agricultural practices such as pruning and  
54 grafting (Hodgetts *et al.*, 2007).

55 While the number of known insect vectors for phytoplasma is limited to the phloem  
56 feeders of the order Hemiptera (Weintraub & Beanland, 2006), there is a broad range  
57 of host plants. Phytoplasma infections have been found in over 700 plant species  
58 (Hoshi *et al.*, 2009). Some of these infections cause severe damage to agricultural  
59 crops with serious economic impact. Some examples are: lethal yellowing of palms  
60 (Harrison *et al.*, 2008), peach X-disease, apple proliferation (Tedeschi *et al.*, 2003)  
61 and Bois Noir (BN), a grapevine phytoplasmosis. The latter is the focus of this study.  
62 Typical BN disease symptoms on *Vitis vinifera* include necrotic leaves with  
63 downward rolled margins, unligified branches and shriveled berries (Alma, 2002).  
64 During winter season the shoots do not lignify and turn black, giving the disease its  
65 name of Bois Noir (Maixner, 1994). The infection usually leads to a significant  
66 decrease in yield (Garau *et al.*, 2007). Due to the growing economic impact of the  
67 disease in European grapevine production, a better understanding of the transmission  
68 and dynamics of this phytoplasma is therefore of great importance.

69 According to current literature, the main vector of BN in Western Europe is the cixiid  
70 plant hopper *Hyalesthes obsoletus* Signoret 1865 (Alma *et al.*, 1987, Maixner, 2006,  
71 Maixner *et al.*, 1995, Sforza *et al.*, 1998). Although *H. obsoletus* feeds on a wide

72 range of herbaceous plants, its life cycle can only be completed on few hosts. The two  
73 most relevant host plants in Germany are stinging nettle (*Urtica dioica* L.) and field  
74 bindweed (*Convolvulus arvensis* L.) (Maixner, 2005). According to a classification  
75 based on the genetic sequences of the elongation factor tu (*tuf*), two main genetic  
76 types can be distinguished: *Candidatus* Phytoplasma solani *tuf*-type a is related to *U.*  
77 *dioica*, while *Ca. P. solani tuf*-type b is associated to *C. arvensis* (Langer & Maixner,  
78 2004). Both strains have grapevine as a “dead end host”, which means that insects can  
79 infect a grape plant, but cannot acquire phytoplasma from infected grape plants (Kaul  
80 *et al.*, 2009). Before the year 2000, *H. obsoletus* was considered a rare species in  
81 Germany, and *C. arvensis* was the major host plant for this epidemic cycle (Sergel,  
82 1986). In the last 20 years, however, *H. obsoletus* was found more frequently on *U.*  
83 *dioica*. It has been speculated that the increasing mean temperatures could be  
84 connected to this host plant shift (Boudon-Padieu & Maixner, 2007). The new host  
85 allowed the insect to colonize new areas (that is where *U. dioica* is present and *C.*  
86 *arvensis* absent); but also increase its population density in areas where it was already  
87 present (Maixner *et al.*, 2007). For these reasons, BN has become an increasing  
88 concern in Germany.

89 Our study is concentrates on the region of Baden. Located in South-West Germany at  
90 the borders of France and Switzerland, Baden is one of the most important areas of  
91 Germany for wine production. Since BN is a relatively new challenge, only few  
92 studies have examined this epidemiological system in this area. Darimont & Maixner  
93 (2001) conducted an analysis of insect infestation in the year 1999 and 2000. Breuer  
94 *et al.* (2008) conducted a monitoring of the occurrence of *H. obsoletus* proving the  
95 insects’ presence in all wine-growing districts, and Panassiti *et al.* (2013) reported the  
96 presence of the insect all over the region. From these studies, we have a basic  
97 understanding of the ecology of the insect vector and disease, but so far there is still  
98 very little known on disease prevalence in the insect population. It would also be  
99 desirable to better understand whether the phytoplasma infection has any  
100 consequences for the vector in terms of body size and thus implicates consequences  
101 for the vector fitness, e.g. on fecundity (Honěk, 1993).

102 To address these questions, we analyzed morphological traits and pathogen infection  
103 using real-time PCR of 125 *H. obsoletus* individuals collected in 45 vineyards across  
104 the region of Baden. The study objectives were i) to examine if morphological traits  
105 (body size and weight) of *H. obsoletus* differ between infected and non-infected  
106 individuals ii) to investigate the prevalence of *Ca. P. solani* in the vector population;  
107 and iii) to identify the predominant *Ca. P. solani* strain in the Baden region.  
108

## 109 **Materials and Methods:**

110 In 2012, 85 vineyards in the Baden region (SW Germany) were selected for a bois  
111 noir (BN) monitoring (Panassiti *et al.*, 2015). The selected vineyards were surveyed  
112 for soil-borne insect vector *Hyalesthes obsoletus* between June and August 2012 and  
113 2013. Yellow sticky traps and the viticulture prediction tool “vitimeteo”  
114 ([www.vitimeteo.de](http://www.vitimeteo.de)) were used to monitor and predict the flight activity of the insects,  
115 in order to guarantee optimal sampling conditions. Sampling of *H. obsoletus*  
116 individuals was performed as described in Panassiti, *et al.* (2013). In brief, in each of  
117 the locations, potential host plants were identified. If *U. dioica* was found, the  
118 sampling consisted of sweeping a sweep net (30 cm diameter) over the selected  
119 plants. If *C. arvensis* was found, suction sampling was performed. Stinging nettle

120 patches were swept 5 times per square meter. Suction sampling was applied for 3  
121 minutes for every square meter of the patch.

122 The collected insects were transported in a cooling box and freeze dried in the  
123 laboratory with a freeze dryer “Christ Alpha 1-2 LDplus” (Martin Christ Freeze  
124 Dryers, Germany). We determined species and gender following the identification  
125 keys of Biedermann & Niedringhaus (2004) with a microscope “Zeiss Stereo  
126 LUMAR 1.2”, (Carl Zeiss, Germany). We measured body length with the software  
127 “Axiovision Rel 4.8”. The length of an individual was defined as the distance from  
128 head to the bottom of the fore-wings (Fig. 2). The samples were then frozen in liquid  
129 nitrogen and conserved at -80°C for further analyses.

130 DNA extraction and quantitative real-time PCR (qPCR) was carried out with the same  
131 procedure for extraction and the same primers, probes and cycle settings for qPCR as  
132 in Fahrentrapp *et al.* (2013). This method utilizes hydrolysis probes specific for  
133 selective DNA fragments of both, phytoplasma types I and II, as well as for insect  
134 DNA. The method allows to detect an infection and to distinguish the phytoplasma  
135 type. Based on the obtained results, we calculated the amount of phytoplasma DNA  
136 relative to the insect DNA, using the method  $2^{-\Delta\Delta Ct}$  as described by Livak &  
137 Schmittgen (2001) for relative quantification of gene expression.

## 138 **Results**

139 We collected 125 *Hyalesthes obsoletus* individuals from the 45 vineyards (Fig. 1). 52  
140 of those were identified as males, 70 as females and 3 were not identifiable due to the  
141 lack of the final abdominal segments. The observed female ratio of 56% is not  
142 significantly different from an even sex ratio ( $p=0.18$  with a binomial test against  $H_0$   
143 = 50%; the 95% confidence interval spans 47% - 65%). Of all 125 individuals, five  
144 (four females and one male) were infected with *Ca. P. solani*, tuf-type a. This  
145 corresponds to a disease prevalence of 4%, with the 95% confidence interval from a  
146 binomial model ranging from 1.3% to 9.2%. The five infected individuals were  
147 caught in different locations spread all over Baden (Fig. 1), with no discernable  
148 spatial pattern. The amount of phytoplasma DNA detected in the samples varied  
149 substantially. The sample with the highest amount of phytoplasma DNA has roughly  
150 17 times more phytoplasma-DNA than the sample with the lowest amount (Table 1).

151 We observed a difference in weight and length between males and females. Females  
152 were in general longer and heavier than males. The average length and dry weight for  
153 females and males was 4.95 (+/-0.31), 4.01 (+/-0.28) mm as well as 1.7 (+/-0.57) and  
154 0.67 (+/-0.31) mg, respectively. Our results for sex ratio, weight and length are also  
155 displayed table 2 and in figures 3 and 4. Due to the low number of infected insects, it  
156 was not possible to draw any conclusions about the influence of infections on insect  
157 length and body mass. The five infected individuals show values that are well within  
158 the range found for non-infected individuals.

## 159 **Discussion and Conclusions**

160 The prevalence of *Ca. P. solani* infection in *H. obsoletus* determined in this study was  
161 surprisingly low. Although we have evidence of infected plants in studied vineyards  
162 (Panassiti, *et al.*, 2015), we only found five infected individuals in 125 samples  
163 analyzed (4%). Previous studies in different areas showed higher infection rates.  
164 Langer & Maixner (2004) observed insect infection rates of 28% and 54% (host plant  
165 *U. dioica*) in two different viticultural areas in Germany. In a study conducted in the

166 north of Italy, Lessio *et al.* (2007) reported a variable infection rate during different  
167 years, reaching up to 80%. Darimont & Maixner (2001), who also sampled in Baden,  
168 but over different years, reported an average infection rate of 23% over several years.  
169 We can only speculate about the reasons for the low disease prevalence in the present  
170 study. The sample size of this study is relatively low, but as the 95% confidence  
171 interval extended only up to 9.2%, the explanation that random variation alone is  
172 causing this low prevalence seems incompatible with the assumption that the true  
173 disease prevalence is around 20%. A plausible explanation is that the sampling  
174 methods used in our study were different from previous studies. In our study,  
175 sampling locations were chosen randomly. In the previously mentioned studies the  
176 site selection criteria were sometimes not clearly described and it could be that  
177 sampling locations were chosen close to vineyards in which the disease has already  
178 been observed. In this case, a higher prevalence would be expected. We see a need for  
179 further studies to resolve the question of the average disease prevalence in the Baden  
180 region.

181 The length measurements of *H. obsoletus* are in agreement with values from the  
182 literature. Alma (2002) described body length of 3.7 - 4 mm and around 5 mm for  
183 males and females, respectively. To our knowledge, no previous studies reported on  
184 the dry body weight of the insects that could be compared to our results. No  
185 significant bias of the sex-ratio was observed in this study, although this could have  
186 been expected for our sampling method: using yellow sticky-traps, the gender ratio  
187 may be biased (Lessio, *et al.*, 2007), because in many Homoptera species males have  
188 a greater flight activity and a higher dispersal rate compared to females (Lessio &  
189 Alma, 2004).

190 In conclusion, our study showed an unexpectedly low prevalence of BN-causing  
191 phytoplasma in individuals of the insect vector *H. obsoletus* caught in Baden. We  
192 were not able to draw any conclusion on differences in size of the individuals due to  
193 the low number of infected individuals.

194 Further surveys with a higher number of specimen, sampling locations and an analysis  
195 of plant material for *U. dioica*, *C. arvensis* and *V. vinifera*, will help to understand if  
196 this low prevalence reflects a true decline of the disease in the vector population, or  
197 whether it can be explained with systematic differences with previous studies.

198

### 199 **Acknowledgments**

200 Phytoplasma infected *H. obsoletus*-derived DNA samples used as positive controls in  
201 qPCR were kindly provided by M. Maixner. We thank Dr. Breuer and the Staatliches  
202 Weinbauinstitut Freiburg, for providing the possibility to conduct this study.

203

204 **Tables**

205

206

207

208

209 Table 1. Morphological measurements qPCR results (DNA folds) for infected

210 *Hyalesthes obsoletus*.

Sample	Sex	Length (mm)	Dry weight (mg)	DNA Folds*	St. Dev.
1114	Female	4.74	1.7	2.6	0.3
1109	Female	4.03	1.7	17.2	0.5
1123	Female	5.19	2.3	3	0.7
1041	Female	4.9	2.1	13.3	0.7
1134	Male	3.48	1.1	1	1

211 \*The DNA folds are calculated using the ratio phytoplasma DNA/insect DNA,  
 212 relative to the sample with the lowest amount of DNA (1134). Relative titers of DNA  
 213 normalized to sample 1134.

214

215

216

217 Table 2. Maximum, minimum and average values of morphological observations

218 grouped by sex of all specimens collected in this study.

219

Measurement	Average value (Std. Dev.)	Max.	Min
Sex ratio (%)	56 (9)		
Male length (mm)	4,01 (0.28)	4.49	3.18
Female length (mm)	4.95 (0.42)	5.7	3.73
Male body mass (mg)	0.67	1.4	0.1
Female body mass (mg)	1.7 (0.57)	2.7	0.2

220

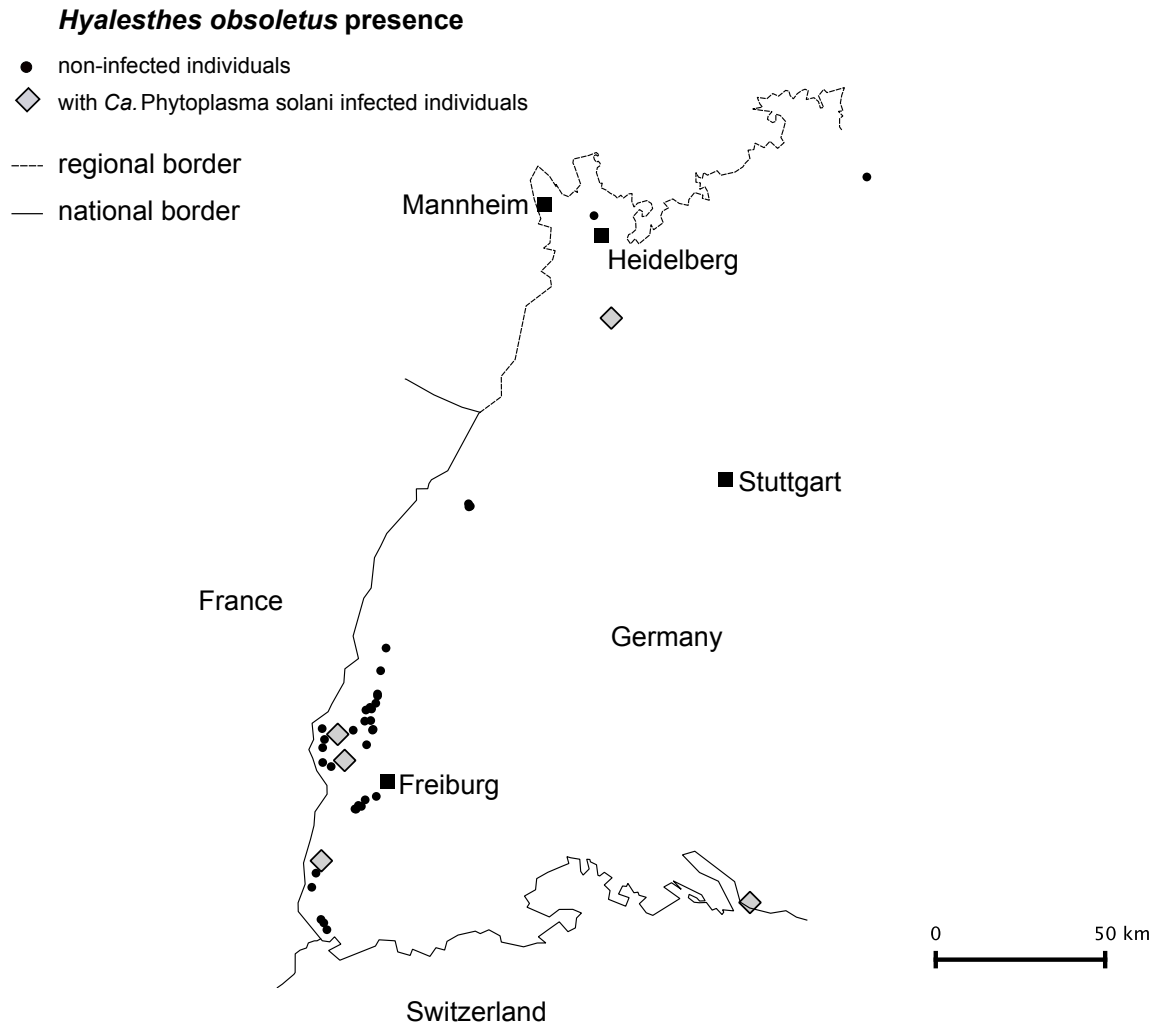
221 **Figures**

222

223 Figure 1. Collection sites in the Baden region. Black dots are the sampling locations.

224 Grey squares represent the sampling locations where infected *H. obsoletus* were

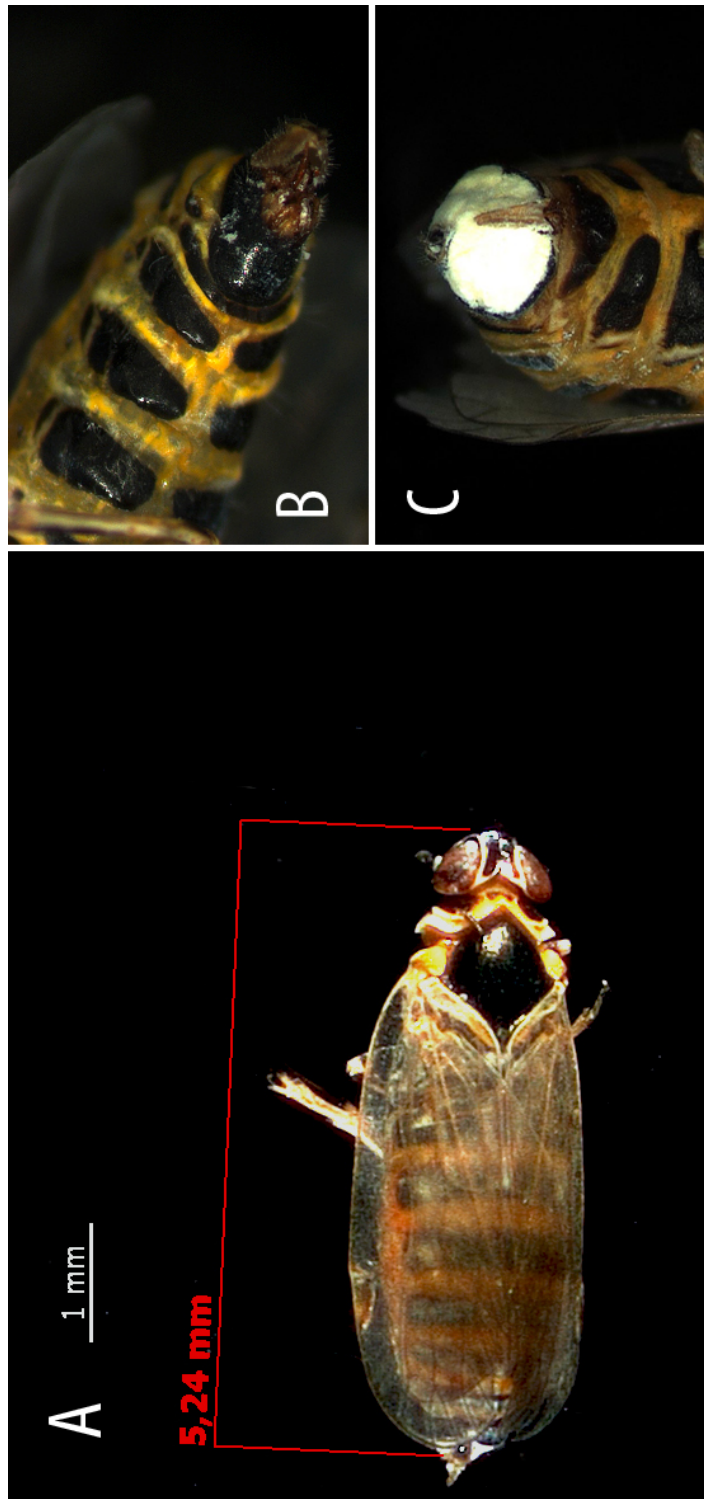
225 found.



226

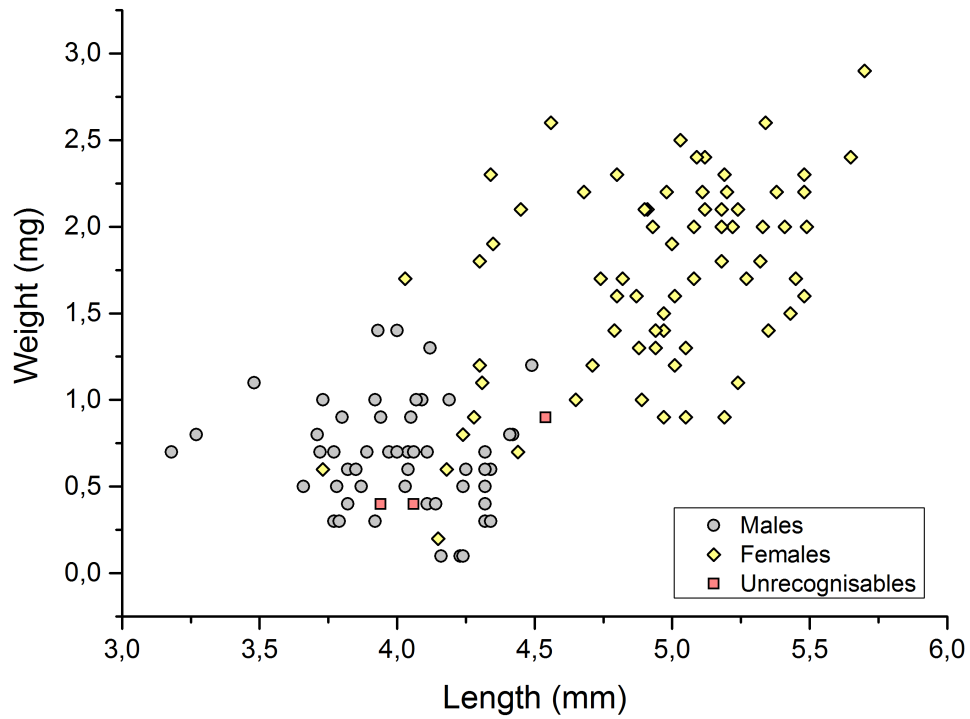
227

228 Figure 2. Magnified photographs of (A) *H. obsoletus*, (B) male genital capsule, and  
229 it's (c) female ovipositor.  
230  
231  
232



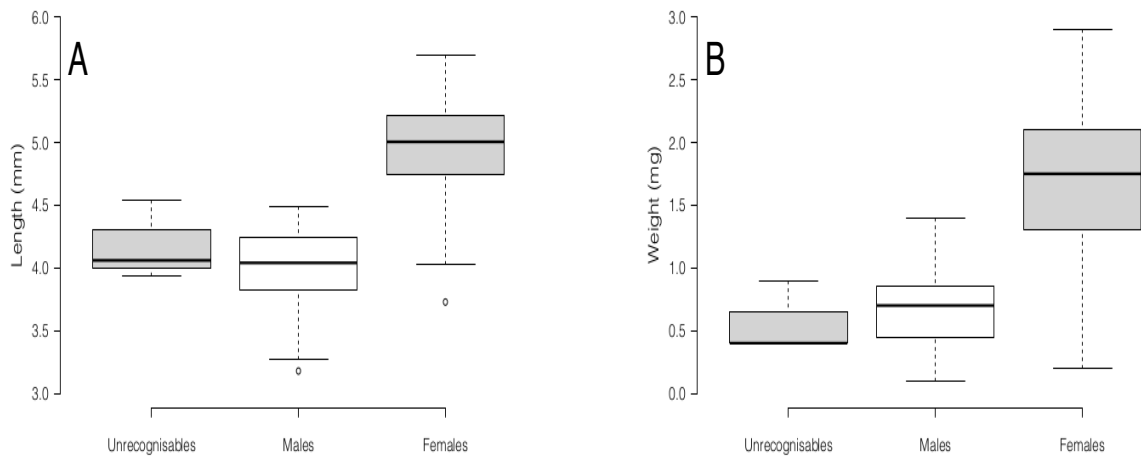


233 Figure 3. Distribution of length and weight for the individuals analyzed. Females are  
234 colored in yellow diamonds, males are represented in red dots and the samples with  
235 the abdomen missing (unrecognisable) are shown in blue squares.



236  
237  
238  
239  
240

Figure 4. A: Box plots of distribution of length (A) and weight (B) of specimen collected in this study.



241  
242

243 **Literature**

244

245

246 Alma, A., 2002. Auchenorrhyncha as pests on grapevine. *Denisia*. 4(176): 531-538.

247 Alma, A., Arnò, C., Arzone, A. Vidano, C., 1987.- New biological reports on

248 Auchenorrhyncha in vineyards pp. 509-516. (Vidano, C. and Arzone, A., Eds)

249 Proceedings of 6th Auchenorrhyncha Meeting, Torino, Italy.

250 Biedermann, R. Niedringhaus, R., 2004.- The planthoppers and leafhoppers of  
251 Germany - Identification key to all species.- WABV-Verlag, Scheeßel, Germany.

252 Boudon-Padieu, E. Maixner, M., 2007.- Potential effects of climate change on  
253 distribution and activity of insect vectors of grapevine pathogens pp. 1-8. Global  
254 warming, which potential impacts on the vineyards?, Dijon, France.

255 Breuer, M., Fahrentrapp, J. Michl, G., 2008.- Geographical distribution of "bois noir"  
256 and *Hyalesthes obsoletus* in Baden (SW Germany). 1st International Bois Noir  
257 Workshop 2008,

258 Christensen, N. M., Axelsen, K. B., Nicolaisen, M. Schulz, A., 2005. Phytoplasmas  
259 and their interactions with hosts. *Trends in Plant Science*. 10(11): 526-535.

260 Darimont, H. Maixner, M., 2001. Actual distribution of *Hyalesthes obsoletus* Signoret  
261 (Auchenorrhyncha: Cixiidae) in German viticulture and its significance as a vector of  
262 Bois noir. *IOBC/WPRS Bulletin*. 24: 199-202.

263 Fahrentrapp, J., Michel, G. Breuer, M., 2013. Quantitative PCR assay for detection of  
264 Bois noir phytoplasmas in grape and insect tissue. *Vitis*. 52(2).

265 Garau, R., Sechi, A., Prota, V. A. Moro, G., 2007. Productive parameters in  
266 Chardonnay and Vermentino grapevines infected with "bois noir" and recovered in  
267 Sardinia. *Bulletin of Insectology*. 60(2): 233.

268 Harrison, N. A., Helmick, E. E. Elliott, M. L., 2008. Lethal yellowing-type diseases of  
269 palms associated with phytoplasmas newly identified in Florida, USA. *Annals of*  
270 *Applied Biology*. 153(1): 85-94.

271 Hodgetts, J., Ball, T., Boonham, N., Mumford, R. Dickinson, M., 2007. Use of  
272 terminal restriction fragment length polymorphism (T-RFLP) for identification of  
273 phytoplasmas in plants. *Plant Pathology*. 56: 357-365.

274 Hogenhout, S. A., Oshima, K., Ammar, E.-D., Kakizawa, S., Kingdom, H. N. Namba,  
275 S., 2008. Phytoplasmas: bacteria that manipulate plants and insects. *Molecular Plant*  
276 *Pathology*. 9(4): 403-423.

277 Honěk, A., 1993. Intraspecific variation in body size and fecundity in insects: a general  
278 relationship. *Oikos*. 483-492.

279 Hoshi, A., Oshima, K., Kakizawa, S., Ishii, Y., Ozeki, J., Hashimoto, M. Namba, S.,  
280 2009. A unique virulence factor for proliferation and dwarfism in plants identified  
281 from a phytopathogenic bacterium. *Proceedings of the National Academy of Sciences*.  
282 106(15): 6416-6421.

283 Kaul, C., Seitz, A., Maixner, M. Johannesen, J., 2009. Infection of Bois-Noir tuf-type-I  
284 stolbur phytoplasma in *Hyalesthes obsoletus* (Hemiptera: Cixiidae) larvae and  
285 influence on larval size. *Journal of Applied Entomology - Zeitschrift Fur Angewandte*  
286 *Entomologie*. 133(8): 596-601.

287 Langer, M. Maixner, M., 2004. Molecular characterisation of grapevine yellows  
288 associated phytoplasmas of the stolbur-group based on RELP-analysis of non-  
289 ribosomal DNA. *Vitis*. 43(4): 191-199.

290 Lessio, F. Alma, A., 2004. Seasonal and daily movement of *Scaphoideus titanus* ball  
291 (Homoptera : Cicadellidae). *Environmental Entomology*. 33(6): 1689-1694.

292 Lessio, F., Tedeschi, R. Alma, A., 2007. Population dynamics, host plants and  
293 infection rate with stolbur phytoplasma of *Hyalesthes obsoletus* Signoret in north-  
294 western Italy. Journal of Plant Pathology. 89(1): 97-102.

295 Livak, K. J. Schmittgen, T. D., 2001. Analysis of relative gene expression data using  
296 Real-Time Quantitative PCR and the 2<sup>-ddcT</sup> method. Methods. 25: 402–408.

297 Maixner, M., 2006.- Grapevine yellows – current developments and unsolved  
298 questions pp. 86-88. 15th Meeting of ICVG, Stellenbosch, South Africa.

299 Maixner, M., 2005.- Risks posed by the spread and dissemination of grapevine  
300 pathogens and their vectors pp. 141-146. Introduction and Spread of Invasive Species,  
301 Symposium Proceedings,

302 Maixner, M., 1994. Transmission of German grapevine yellows (Vergilbungskrankheit)  
303 by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). Vitis. 33(2):  
304 103-104.

305 Maixner, M., Ahrens, U. Seemüller, E., 1995. Detection of the German grapevine  
306 yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a  
307 specific PCR procedure. European Journal of Plant Pathology. 101: 241-250.

308 Maixner, M., Johannesen, J., Michel, K., Lux, B. Seitz, A., 2007. Host plant specificity  
309 of *Hyalesthes obsoletus* and consequences for “bois noir” epidemiology. Bulletin of  
310 Insectology. 60(2): 399-400.

311 Panassiti, B., Breuer, M., Marquardt, S. Biedermann, R., 2013. Influence of  
312 environment and climate on occurrence of the cixiid planthopper *Hyalesthes obsoletus*,  
313 the vector of the grapevine disease ‘bois noir’. Bulletin of Entomological Research.  
314 103: 621–633.

315 Panassiti, B., Hartig, F., Breuer, M. Biedermann, R., 2015. Bayesian inference of  
316 environmental and biotic factors determining the occurrence of the grapevine disease  
317 ‘bois noir’. Ecosphere. in press.

318 Sergel, R., 1986. Ein weiterer Nachweis der Cixiide *Hyalesthes obsoletus* Signoret in  
319 Franken (Homoptera: Auchenorrhyncha: Fulgoroidea). Abhandlungen des  
320 Naturwissenschaftlichen Vereins Würzburg. 25: 81-82.

321 Sforza, R., Clair, D., Daire, X., Larrue, J. Boudon-Padieu, E., 1998. The role of  
322 *Hyalesthes obsoletus* (Hemiptera: Cixiidae) on the occurrence of bois noir of  
323 grapevines in France. Journal of Phytopathology. 146: 549-556.

324 Tedeschi, R., Visentin, C., Alma, A. Bosco, D., 2003. Epidemiology of apple  
325 proliferation (AP) in northwestern Italy: evaluation of the frequency of AP-positive  
326 psyllids in naturally infected populations of *Cacopsylla melanoneura* (Homoptera:  
327 Psyllidae). Annals of Applied Biology. 142(3): 285–290.

328 Weintraub, P. G. Beanland, L., 2006. Insect vectors of phytoplasmas. Annual Review  
329 of Entomology. 51: 91-111.

330

331

332