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Seasonal progression of sex ratio and phytoplasma infection in *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae)

F. Lessio, R. Tedeschi, M. Pajoro and A. Alma*

Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali (Di Va PRA), Entomologia e Zoologia applicate all'Ambiente 'C. Vidano', University of Turin, via L. da Vinci 44, 10095 Grugliasco (TO), Italy

Abstract

The differences between the seasonal occurrence and likelihood of being infected by FD phytoplasmas, of male and female *Scaphoideus titanus* Ball, were investigated. Sex ratio (male: female) was calculated by counting males and females sampled by means of yellow sticky traps and sweep-nets and from adults derived from hatched eggs in field-collected grapevine wood. PCR essays were performed to test differences in infection between genders. Overall, the sex ratio on sticky traps was significantly more male biased (1.99 : 1) if compared to net sweeping (0.62 : 1) and laboratory rearing (0.60 : 1). The peak of male presence was recorded in the middle of July in laboratory rearing and sweep net, and in the middle of August on sticky traps; the maximum presence of females was detected at the end of July in laboratory rearing, and at the end of August in sweep net samplings and on sticky traps. The seasonal sex ratio was more male biased at the beginning in laboratory rearing (1.50 : 1) and sticky traps (9 : 1), and then decreased in favor of females at the end of the sampling period, both in laboratory rearing (0.17 : 1) and in sticky traps (0.07 : 1). This trend was significantly less skewed, although similar, in sweep net samplings that recorded a sex ratio of 1 : 1 and 0.16 : 1 at the beginning and at the end of the sampling period, respectively. Concerning phytoplasma detection, an interaction between gender and sampling period was observed, the males showing a peak of infected individuals later in the season (35%). Some possible behavioral explanations of the data obtained are given.

Keywords: grapevine, Flavescence dorée, leafhopper vector, seasonal sex ratio change, phytoplasma detection rates

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Introduction

Flavescence dorée (FD), caused by EY phytoplasmas (16SrV, subgroups C and D) is a serious disease of grapevine that occurs in Italy, France, Spain, Serbia, Switzerland and Slovenia (Boudon-Padieu, 2003; Duduk *et al*., 2004; Schaerer *et al*., 2007; Seljak, 2008). It is transmitted by *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae), a nearctic species introduced into Europe in the 1950s and now present in France, Italy, Spain, Switzerland, Croatia, Slovenia, Serbia, Portugal (Alma, 2004), Austria (Zeisner, 2005), Bosnia-Herzegovina (Delić *et al*., 2007) and Hungary (Dér *et al*., 2007). This leafhopper is monophagous on *Vitis* spp., including European (*V. vinifera* L.) and American (e.g. *V. rupestris* Scheele and *V. cordifolia* Michx) grapevine (Maixner *et al*.,

*Author for correspondence
Fax: +39 011 6708535
E-mail: alberto.alma@unito.it

1993; Beanland *et al.*, 2006). Overwintering occurs as eggs laid beneath the bark (Vidano, 1964) and nymphs can acquire phytoplasmas by feeding on infected plants, whereas adults are responsible for the transmission of the disease, as they become infective after a 5–6 week latency period from the time of exposure of leafhopper to infected plants (Bressan *et al.*, 2006). Adults usually appear at the beginning of July and can be observed until the middle of October (Vidano, 1964); the flight activity is mainly crepuscular (Lessio & Alma, 2004a) and is restricted to the grapevine's canopy (Lessio & Alma, 2004b). Previous research has proven that the highest infection rate occurs in the second part of the summer, that is, during September and October (Bressan *et al.*, 2006). On the whole, sticky traps tend to catch more males (Lessio & Alma, 2004b), but it is not clear if this is due to differences in behavior between genders or reflects the effective sex ratio. This research focuses on the seasonal presence of *S. titanus* males and females, and on its consequences on the risk of transmitting FD phytoplasmas during the season.

Materials and methods

Field collections

Data were collected during 2006 in three vineyards of the province of Asti (Piedmont, NW Italy). The vineyards were planted with the 'Barbera' grapevine variety. They were approximately 30 years old and pruned per the Guyot method. Their size was approximately 2000 m^2, and they were isolated from other vineyards. They presented a high incidence of FD, and no insecticides were used.

Adults were obtained in three different ways: rearing of adults from field-collected eggs, sweep-net samplings and yellow sticky traps.

Egg sampling was accomplished by collecting pruned grapevine canes of at least two-year-old wood, which is chosen by *S. titanus* for egg laying. Samples were collected during the winter from the middle of January to the end of February. In each vineyard, approximately 12 kg of wood were randomly collected. Canes were cut into 20–40 cm long pieces, stored in plastic crates and brought to the laboratory. Here, they were kept outdoors at the same environmental conditions (temperature and relative humidity) as the investigated vineyards until the end of April, so to complete the degree days for egg hatching, and they were periodically sprinkled with water to avoid the dehydration of the eggs. Afterwards, canes were put into a mass rearing cage (120 × 120 × 100 cm) made of a metal frame covered with an insect-proof mesh that contains a zip opening. Healthy vine plants, approximately 50 cm high and potted in 20 cm diameter pots, were put inside the cage and periodically sprinkled. The mass rearing was kept outside, in the same conditions as the stored canes. After hatching, *S. titanus* nymphs immediately jumped onto vine leaves and started to feed and molt. Plants were changed every two weeks, or when too many nymphs were present. When nymphs reached the 5th instar, they were collected with an aspirator and moved into smaller cages (20 × 20 × 40 cm) made of Plexiglas and insect-proof mesh, with a sleeve to introduce and remove insects, a square lid and a hole in the base in which a potted healthy vine plant was introduced. Ten cages were used, and each cage hosted up to 30 *S. titanus* nymphs. Adults were picked up 48 h after their emergence and frozen (−20°C) before sexing.

For field sampling, we used yellow sticky traps (Glutor®), 20 × 30 cm, coated with glue on both sides. Traps were placed into the vineyards by fastening them to the wires within the grapevine canopy at a 150 cm height from ground level. Traps were arranged, three per vineyard, along a diagonal pattern from one edge to another, in order to cover the whole vineyard size, and were changed every 10–20 days from the beginning of July to the middle of October. Removed traps were wrapped in a transparent plastic film, labeled and brought to the laboratory where they were stored inside a freezer (−20°C). *S. titanus* adults were also sampled by means of a sweep net (40 cm dia.) directly on the vine canopy; samplings were carried out every 5–10 days during the morning (9:00–11:00 am) and lasted approximately 60 min per sampling unit. During this period of time, up to 15 vine rows, 50 m long, were sampled in each vineyard; adults were collected from the sweep net with an aspirator and kept in glass test-tubes inside a cool bag (+5°C) before they were brought to the laboratory.

In the laboratory, the *S. titanus* adults, collected with different sampling methods, were counted and sexed by observing the external gonapophises with a 20× stereomicroscope. Leafhoppers were detached from sticky traps with a drop of Bio-Clear (Bio-Optica, Milano, Italy).

Laboratory analyses

In order to study the influence of time and gender on FD infection rates, *S. titanus* adults were analyzed by means of molecular techniques to detect the presence of FD phytoplasmas (16SrV). Total DNA was extracted, following a procedure previously described for leafhoppers (Marzachì *et al.*, 1998), from single *S. titanus* individuals collected by mean of sticky traps or sweep net and analyzed. Only individuals that seemed to be recently captured on sticky traps were analyzed. Adults obtained via rearing of eggs were not analyzed, since 16SrV phytoplasmas are not transmitted to the progeny (Alma *et al.*, 1997). Samples were amplified in direct PCR with the group specific ribosomal primer pair fAY/rEY (Marcone *et al.*, 1996; Marzachì *et al.*, 2001). PCR products were analyzed by eletrophoresis through 1% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

Statistical analyses

The null hypotheses were stated as follows: sex ratio (SR) of *S. titanus* is independent from the sampling method and from the seasonal period of collection; and FD infection is not dependent from *S. titanus* gender and seasonal period of collection. Data were analyzed via binary logistic regression (Forthofer *et al.*, 2007). For testing the first two hypotheses, *S. titanus* gender was used as the binary dependent variable (male = 1; female = 0), while as explanatory variables we considered: the sampling method (sticky traps, sweep net sampling and pruned canes) as a categorical (dummy) variable, fixing pruned canes as the reference variable; and the period of adults' emergence (i.e. days after the emergence of the first adults) as a continuous variable. For testing the last two hypotheses, FD infection of leafhoppers was considered as the dependent variable (infected = 1; healthy = 0), whereas the period of adults' emergence and leafhoppers'

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Table 1. Parameter coding of explanatory dummy variables used in logistic regression.

Experiment	Explanatory dummy variable	Categories	Frequency	Parameter coding (1)	Parameter coding (2)
Seasonal progression of SR	Sampling method (SM)	Egg rearing	278	0	0
		Sweep Net	612	1	0
		Sticky traps	1998	0	1
Seasonal progression of FD infection	Gender (G)	Females	82	0	–
		Males	100	1	–

gender (male = 1; female = 0) were the explanatory variables. In both cases, the interaction between the independent variables was also calculated. Variables were inserted into the model via a backwards stepwise likelihood ratio method. Coding of dummy variables is represented in table 1. Overall significance of the model was tested via a Hosmer-Lemeshov test. All data were analyzed with the SPSS® 12.0 statistical package.

Results

Seasonal trend of sex ratio

S. titanus eggs started to hatch on 15th May and hatching kept going on for almost 30 days. The first adults emerged from the rearing on the 18th of July when the peak of males was also reached, whereas the peak of females was recorded ten days later. No adults emerged from the rearing after the 22nd of August. A total of 278 adults was reared, the SR being 0.60 : 1 (105 males and 173 females); this value was comparable with the SR obtained via net sweeping, 0.62 : 1 (235 males, 377 females), whereas the SR obtained with yellow sticky traps was much more male biased, being 1.99 : 1 (1331 males, 667 females). Field captures of *S. titanus* adults with sticky traps started on the 28th of July and ended on the 26th of September, whereas no captures by means of a sweep net were obtained after the beginning of September. The flight peak was detected with yellow sticky traps on the 12th of August for males, and on the 27th of August for females. On the other hand, the maximum presence of *S. titanus* adults caught using the sweep net was detected on the 23rd of July for males, and on the 1st of September for females (fig. 1).

The maximum bias in favor of males was observed on the 18th of July in egg rearing (1.5 : 1) and in sweep net samplings (1 : 1) and on the 28th of July on sticky traps (9 : 1), whereas the maximum bias in favor of females was recorded on the 22nd of August in laboratory rearing (0.17 : 1) and sweep net sampling (0.16 : 1), and on the 26th of September on sticky traps (0.07 : 1) (fig. 2). Binary logistic regression equation provided a good fit with the observed data of SR of *S. titanus* (Hosmer-Lemeshov test, $\chi^2 = 4.58$, 6 df, $P = 0.60$). The model classified correctly 74.0% of the observations (54.3% for females, 84.5% for males) vs. 65.1% classified from the model with intercept only ($\chi^2 = 386.02$, 5 df, $P < 0.001$). The relationship was significant (Wald test, $P < 0.05$) for the sampling period and sampling with sticky traps, SM (2), but not for sampling with the sweep net, SM (1) (table 2, fig. 2). The interaction between sampling period and sampling method was significant for SM (1) but not for SM (2).

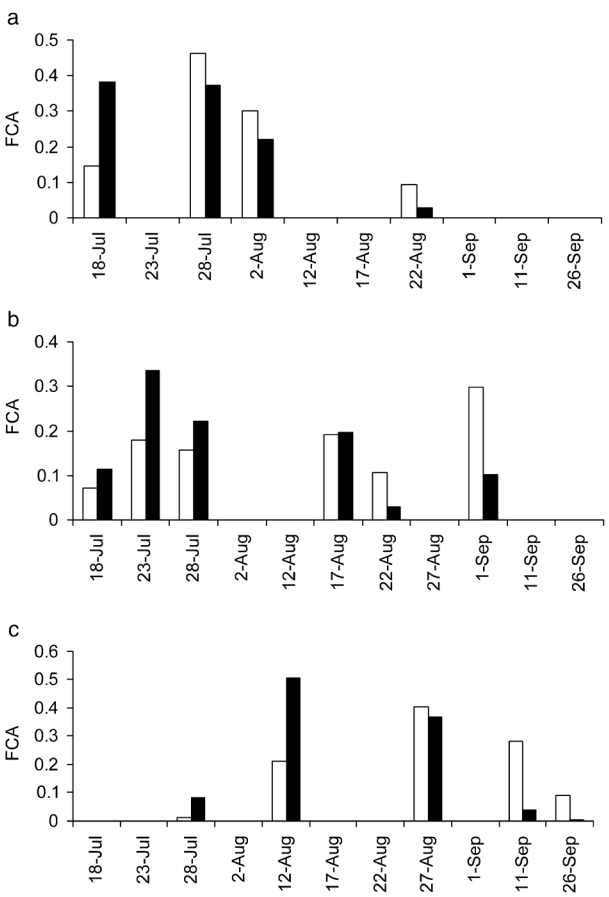

Fig. 1. Seasonal frequency of *S. titanus* adults obtained with different sampling methods: (a) egg rearing, (b) sweep net and (c) sticky traps. FCA, frequency of captured adults (adults captured per sampling date/total adults captured) (□, females; ■, males).

Therefore, the final model included as explanatory variables SM (2), SP and SP × SM (1). The SR of *S. titanus* is significantly related to the seasonal period of sampling (the odd ratio of having a male is 0.93 per day of sampling) and is more male biased in sticky traps captures than in sweep net captures and egg rearing (the odd ratio of having a male is 29.82 in sticky traps), and the decrease of males during the season is less evident in sweep net captures than in sticky traps captures and egg rearing (the odd of having a male is

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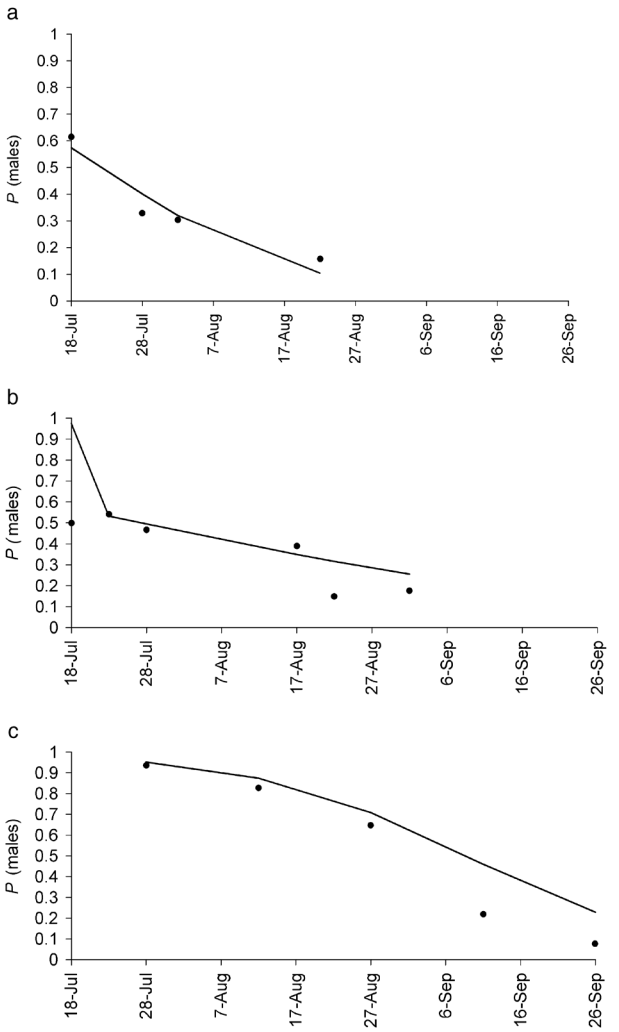

Fig. 2. Sex ratios of *S. titanus* observed at different periods fitted with those predicted by binary logistic regression equations for (a) egg rearing, (b) sweep net and (c) sticky traps. *P* (males), probability of having males (—, predicted; ●, observed).

1.04 per day if sampling is done with sweep net rather than egg rearing or sticky traps).

Seasonal trend of phytoplasma infection

On the whole, 182 *S. titanus* adults were tested for the presence of phytoplasmas (100 males and 82 females). Overall infection rate with FD phytoplasma was 15.3% in males and 9.7% in females; the highest rate was recorded at the end of August when 35% of males and 11% females were found FD positive. Binary logistic regression provided a good fit with observed data (Hosmer-Lemeshov test, $\chi^2 = 2.01$, 4 df, $P = 0.73$). However, only the interaction between the two explanatory variables provided a significant relationship with FD infection; the odds of having a FD positive *S. titanus* male is increased by a coefficient of 1.03 per day (table 3, fig. 3). The model that included the explanatory variables (gender and sampling period, and the interaction between gender and sampling period) predicted correctly the status of FD phytoplasma infection (positive or negative) in 87% of *S. titanus* individuals included in the data set; however, the same result was obtained from the model without the explanatory variables and with the intercept only (i.e. a model that classifies all the 182 subjects of the data set as not infected from FD independently from gender and sampling period) ($\chi^2 = 6.71$, 1 df, $P = 0.01$). In conclusion, FD infection seems to increase during the season only in males, but these data must be considered with caution.

Discussion

Data obtained from laboratory rearing showed a 0.6 : 1 SR for *S. titanus* that should reflect the real SR of the species. The SR calculated with net sweeping was not significantly different. On the other hand, yellow sticky traps caught significantly more males; this is probably due to an overall higher mobility of males, in order to mate. It is known that *S. titanus* females mate only once, whereas this is not clear for males, which might move on to mate again (Mazzoni *et al.*, 2008), and this could explain a female biased SR on a egg rearing basis. Concerning the SR calculated in different periods of the year, a decrease of males' percentage is always evident; males seem to hatch earlier than females. The trend of sticky traps is not different from that detected with egg rearing; on the other hand, the probability of having males decreases significantly in egg rearing from that detected with sweep net samplings. According to Vidano (1964), adults live, at most, a month. However, some individuals might live longer, in which case sweep net samplings made in the late part of the season may catch older males, too; whereas the emergence peak of males might coincide with their flight peak, resulting in no difference between seasonal trend of SR calculated via egg rearing and sticky traps. On the other hand, females might be more active at the end of the season due to their need to find ovoposition sites, and this could result in a female biased SR on stick traps. Females might also have a longer lifespan than males, and this could explain why few males are caught at the end of the season.

Many leafhoppers are known to present an overall male biased SR calculated on a trap catch basis, compared to a 1 : 1 SR on plants, and this is often indicated as local movement rather than migration. *Spissistilus festinus* (Say) (Membracidae) showed a male rate of 89–96% on sticky traps and of 58–67% in sweep net samplings (Johnson & Mueller, 1990). The same behavior was found in *Graminella nigrifrons* Forbes, whose males were more abundant in sticky traps (Rodriguez *et al.*, 1992). *Circulifer haematoceps* (Mulsant & Rey) showed a male rate of 85% on sticky traps and only 26% in D-Vac samplings. However, this species is considered a nocturnal migrant and unmated females might not have been caught during daytime samplings (Kersting & Baspinar, 1995). Males of *Macrosteles quadrilineatus* Forbes were also more engaged in local movements, and SR was 3 : 1 on sticky traps and 1 : 1 on plants. Moreover, laboratory experiments demonstrated how in this species' vertical flights were performed mainly by unmated females, whereas mated females were more sedentary, in which case a real migratory syndrome is likely (Hoy *et al.*, 1999).

These last two studies are consistent with the oogenesis and migration syndrome theory that tells how movement

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Table 2. Logistic regression of sex ratio of *S. titanus* on sampling method (SM) and sampling period (SP). Explanatory variables were inserted into the model via a likelihood ratio stepwise method. Coding of dummy variables: (1), sweep net; (2), sticky traps; given egg rearing as the reference category (see also table 1 for details).

Step	Independent variable	B	E.S.	Wald	df	*P*	Odd ratio
1	SM			12.99	2	0.001	
	SM (1)	−0.82	0.62	1.76	1	0.18	0.43
	SM (2)	3.40	1.31	6.73	1	0.001	29.82
	SP	−0.07	0.02	15.72	1	<0.001	0.93
	SM × SP			6.39	2	0.04	
	SM (1) × SP	0.04	0.02	4.24	1	0.04	1.04
	SM (2) × SP	−0.01	0.03	0.03	1	0.87	0.99
	Constant	1.70	0.56	9.33	1	0.002	5.48

Table 3. Logistic regression of FD infection of *S. titanus* on gender (G) and sampling period (SP). Explanatory variables were inserted into the model via a likelihood ratio stepwise method. Coding of dummy variable G: females were chosen as the reference category (see also table 1 for details).

Step	Independent variable	B	E.S.	Wald	df	*P*	Odd ratio
1	SP	0.008	0.02	0.14	1	0.71	1.01
	G	−0.43	1.12	0.15	1	0.70	0.65
	G × SP	0.03	0.03	1.45	1	0.23	1.04
	Constant	−2.53	0.92	7.54	1	0.006	0.08
2	G	−0.74	0.74	1.01	1	0.32	0.48
	G × SP	0.04	0.02	6.21	1	0.01	1.04
	Constant	−2.23	0.37	35.73	1	0.000	0.11
3	G × SP	0.03	0.01	7.00	1	0.008	1.03
	Constant	−2.45	0.33	55.51	1	0.000	0.09

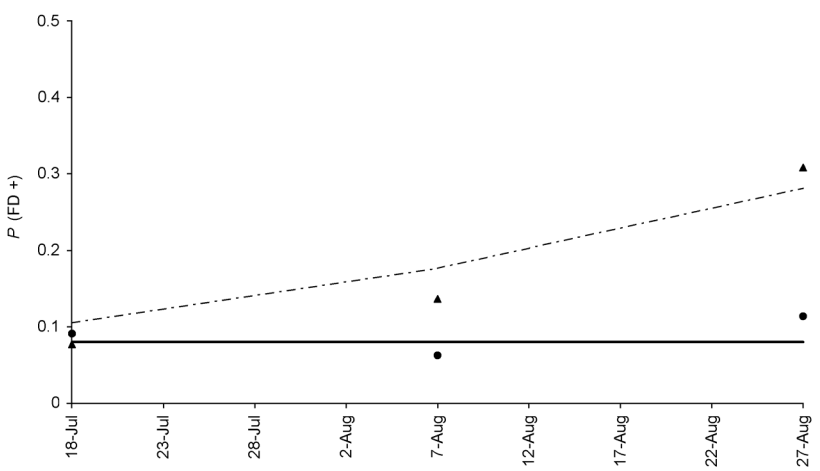

Fig. 3. Progression of FD infection in *S. titanus* observed at different sampling dates fitted with those predicted by binary logistic regression equations. *P* (FD+), probability of having FD positive individuals [—, females (predicted); •, females (observed); –·–·–, males (predicted); ▲, males (observed)].

is regulated by the juvenile hormone (Dingle, 2000). Differences in flight behavior between genders can, therefore, suggest the existence of a migratory syndrome. For instance, it has been demonstrated how females of some *Dalbulus* species are more likely to fly in daytime during autumn and winter because they rely on prevailing winds for migration, whereas during the remaining part of the season, when they are more sedentary, their flight activity is strongly crepuscular (Taylor *et al*., 1993). A female-biased SR at the beginning of the growth cycle, due to the presence of immigrant females, was also demonstrated for the potato leafhopper, *Empoasca fabae* (Harris), another migratory species (Emmen *et al*., 2004). Consistently, *M. quadrilineatus*, another migrant species, present a female-biased SR at the

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beginning of the season on lettuce (Beanland *et al*., 2005). On the contrary, both genders of *S. titanus* have a strongly crepuscular flight activity, and they are therefore unlikely to be carried by the wind (Lessio & Alma, 2004a); moreover, although *S. titanus* adults are tightly bound to the vine's canopy, males seem more likely than females to perform a vertical flight outside the boundary layer (Lessio & Alma, 2004b). This aspect suggests that the dispersal of *S. titanus* is not due to a migratory syndrome but to local movements that seem to occur earlier for males and later for females; whereas a real peak of emergence can be detected at the beginning of the season, both for males and females. Concerning *S. titanus*, it may be concluded that males are more likely to perform local movements, particularly at the beginning of the season when they are also more abundant. Higher female captures at the end of the season also may be due to local movements; and a migratory syndrome is unlikely, considering that this species is bound to a highly persistent host, such as grapevine, and does not need to look for new host plants during the season (Denno *et al*., 2000).

FD infection of *S. titanus* adults increased in males during the season. A similar pattern was found in New York (Maixner *et al*., 1993), but the phytoplasmas detected were the agents North American Grapevine Yellows (NAGY), belonging to the groups of the Aster yellows (16Sr I, subgroup A), and of the X disease (16Sr III, subgroup I) (Beanland *et al*., 2006). Our results should also be considered with caution since fewer males were found and tested at the end of the season. Phytoplasma acquisition in *S. titanus* occurs in the 3rd nymphal instar (Bressan *et al*., 2006) and, therefore, should not be influenced by gender. Therefore, the movement of both sexes might be the major aspect influencing disease transmission. In the early part of the season, males are more likely to transmit the disease as they are more engaged in local movements, whereas females seem to be more sedentary and seldom move to other vine plants. On the other hand, at the end of the season, females are more active and are, therefore, more likely to transmit FD phytoplasmas. Since the phytoplasma concentration in vine plants is higher at the end of the season, a higher percentage of leafhoppers is likely to be infective in this period, when residual effect of insecticides might also be over. It has been proved that, in laboratory conditions, neonicotinoids are more effective than organophosphates in long-term prevention from the infection of daisy plant with Chrysanthemum yellows phytoplasma by means of *Macrosteles quadripunctulatus* (Kirschbaum) (Saracco *et al*., 2008). Since the second treatment of grapevine against *S. titanus* is generally performed with organophosphates, the same results may be obtained. Finally, it has yet to be proved if a difference in the transmission efficiency is present between *S. titanus* males and females. For instance, such a difference was proved in *M. quadrilineatus*, where it was found that the females were more infective (Beanland *et al*., 1999).

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