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STUDIES ON *LOBESIA BOTRANA* AND *SCAPHOIDEUS TITANUS* FOR THEIR SUCCESSFUL MANAGEMENT IN ORGANIC AND CONVENTIONAL VITICULTURE

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January 2nd, 2013 Haya AbouAssaf

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Riassunto

Lobesia botrana Den & Shiff (Lepidoptera: Tortricidae) e *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) sono considerati tra i parassiti animali più importanti della viticoltura europea. *Lobesia botrana* oltre che nel del bacino del Mediterraneo, suo areale di origine, è attualmente presente in Giappone, America Latina e California, mentre lo *Scaphoideus titanus* nativo del Nord America è ampiamente diffuso in Europa. Entrambi gli insetti sono presenti nelle regioni del Nord-est Italia. La loro importanza deriva dal danno quali-quantitativo che arrecano alla produzioni di uva. Il danno è causato per via diretta dall'attività trofica delle larve di *L. botrana* delle infiorescenze e degli acini, o indirettamente dallo *S. titanus* attraverso la trasmissione del fitoplasma agente della Flavescenza dorata. A causa di ciò, sono sempre considerati nei disciplinari di difesa delle regioni italiane. Essi vengono principalmente controllati tramite l'applicazione di insetticidi; recentemente per *L. botrana* si sta diffondendo l'impiego della tecnica della confusione sessuale. La recente Direttiva Europea 128/2009 sull'uso sostenibile dei pesticidi in agricoltura, la comparsa di ceppi di insetti resistenti ai pesticidi di sintesi, la diffusione di queste due specie in nuove regioni e le recenti problematiche legate al cambiamento climatico, impongono una migliore conoscenza della biologia ed ecologia di questi artropodi al fine di un loro controllo più efficace e compatibile con la salute umana e l'ambiente

Una comprensione più completa della fenologia dell'insetto e del suo comportamento potrebbe essere il primo passo per la costruzione di una strategia di controllo coerente e di successo. Questa è la motivazione principale per la quale è stata effettuata questa ricerca. Nel presente lavoro è stata indagata la fenologia(I) e la struttura genetica di popolazioni (II) di *L. botrana* e per *S. titanus* la fenologia (III), il comportamento (IV) e l'interazione con i fitoplasmi agenti dei Giallumi della vite (V).

(I) La fenologia delle tignole dell'uva *L. botrana* ed *Eupoecilia ambiguella* è stata indagata in diverse località in Veneto durante il triennio 2010 - 2012. I risultati mostrano una netta preferenza per *E. ambiguella* delle aree collinari e per *L. botrana* delle zone di pianura. Il numero di generazioni annuali è risultato variabile tra i diversi siti e tra i diversi anni. Generalmente nel 2011 è stato evidenziato un anticipo di voli per entrambi i carpofagi rispetto al 2010 e 2012. Per *E. ambiguella* sono stati osservati dai 2 ai 3 voli. Per quanto riguarda *L. botrana* sono stati registrati dai 2 ai 4 voli. In alcune località, si è avuto un lungo periodo di catture che ha reso difficile stabilire se si erano verificati uno o due voli sovrapposti. Inoltre, a fronte dei quattro voli che sono stati notati in alcune zone, nessuna prova per la quarta generazione è stata fornita. La temperatura è stata

trovata per avere un'influenza sul secondo volo di *E. ambiguella* e l'inizio di questo volo per *L. botrana.*

(II) La struttura genetica delle popolazioni di *L. botrana* è stata indagata in quanto, nonostante l'importanza economica della tignoletta dell'uva, poco è noto dal punto di vista genetico su questo insetto. Al fine di indagare la variazione interspecifica e la struttura genetica di 16 popolazioni di *L. botrana* provenienti dal Bacino del Mediterraneo e dal Medio Oriente, sono stati analizzati sei loci microsatelliti. I risultati hanno mostrato moderati livelli di differenziazione genetica tra le diverse popolazioni. Una elevata eterozigosi è stata notata nella maggior parte delle popolazioni probabilmente a causa dell'accoppiamento tra individui di diverse popolazioni. Le popolazioni provenienti dal Medio Oriente e dalla Spagna appartengono a due diversi gruppi genetici, mentre le popolazioni provenienti dalla Germania e dall'Italia sono risultate mescolate tra loro . Nessun effetto chiaro riferimento geografico è stato rilevato sulle diverse popolazioni studiate. Questi dati sono rilevanti per lo sviluppo di strategie di controllo dei parassiti, in quanto lo studio del flusso genico delle popolazioni potrebbe aiutare a conoscere la distanza entro la quale la popolazione riesce a disperdersi, e di conseguenza capire la scala spaziale entro la quale le tecniche di prevenzione potrebbero essere efficaci.

(III) La fenologia di *S. titanus* è stata indagata in diverse località Venete nel triennio 2010-2012, al fine di conoscere il momento migliore per applicare le diverse strategie di difesa. I dati raccolti hanno dimostrato che le ninfe di *S. titanus* compaiono in maggio, mentre gli adulti è sono presenti, tra la terza decade di giugno - prima metà di luglio fino alla fine di settembre - primi di ottobre. In generale i diversi stadi di sviluppo dell'insetto sono comparsi prima nel 2011 e nel 2012 rispetto al 2010 con alcune varianti tra i diversi siti a seconda dell'altitudine e dell'esposizione dei vigneti. La temperatura ha influito sulla comparsa e sullo sviluppo degli stadi giovanili e la fenologia delle ninfe sembra essere sincrona con lo stadio di sviluppo della pianta ospite. Per quanto riguarda gli adulti, è emerso che ci sono altri fattori oltre la temperatura che possono influenzare la fenologia, come ad es. l'altitudine ed il fotoperiodo.

(IV) La preferenza a diverse cultivar di uva, ed il ruolo degli stimoli olfattivi nel riconoscimento della pianta ospite, sono stati indagati in *Scaphoideus titanus*, in quanto questi parametri comportamentali non sono finora ben noti e spiegati per questa cicalina vettrice. L'attrattività verso i diversi stadi di sviluppo di *S. titanus* è stata indagata su Chardonnay, Cabernet Franc, Merlot e Glera. Inoltre, germogli di vite sani ed infetti dai fitoplasmi agenti dei Giallumi della Vite (Flavescenza dorata e Legno nero) sono stati utilizzati per determinare la capacità della cicalina di distinguere i loro diversi stimoli olfattivi. I risultati di questo studio hanno mostrato che la

preferenza di *S. titanus* per le diverse cultivar indagate varia a seconda dell'età. Chardonnay è risultato attrattivo sulle neanidi e sulle ninfe, mentre il Cabernet Franc è risultato attrattivo per neanidi ed adulti. Per quanto riguarda gli stimoli olfattivi, *S. titanus* è risultato essere in grado di distinguere tra un germoglio di vite sano ed infetto da fitoplasmi dei giallumi, mentre non è in grado di distinguere i due diversi fitoplasmi agenti della FD e del LN. Questi risultati fornendo un aiuto nella comprensione del comportamento degli insetti e la loro distribuzione all'interno dei vigneti, potrebbero contribuire allo sviluppo di utili strumenti per le strategie di monitoraggio e di controllo.

(V) L'effetto dei fitoplasmi agenti dei Giallumi della Vite sulla longevità e sulla sopravvivenza di *Scaphoideus titanus* è stata studiata utilizzando foglie di vite infette come fonte di infezione da fitoplasmi. Oltre alla Flavescenza dorata (FD) è stato indagato anche il fitoplasma agente del Legno nero (BN) in quanto la sua presenza è in aumento in molte regioni italiane e perché è stato rilevato all'interno di *S. titanus*. Inoltre, anche se *S. titanus* non è coinvolto nella trasmissione e diffusione del BN il fitoplasma agente del giallume potrebbe avere un effetto sulla vita dell’insetto. Ninfe appena nate sono state raccolte da germogli di vite asintomatici e allevate artificialmente in condizioni controllate su foglie sane, infette da FD o LN. I risultati hanno mostrato che le ninfe allevate su foglie infette da fitoplasmi, sono sopravvissute di meno e sono diventate adulti in più giorni rispetto a quelle allevate su foglie sane. Le analisi di rilevamento fitoplasmi confermato la capacità di *S. titanus* ad acquisire l'agente causale della BN.

Summary

Lobesia botrana Den & Shiff (Lepidoptera: Tortricidae) and *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) are considered major pests in European viticulture. *Lobesia botrana* is native from the Mediterranean basin and now is present in America while *Scaphoideus titanus* native from North America is an exotic pest for Europe. They are both present in north-eastern Italy. Their importance comes from the big damage they cause to the quality and the quantity of grape production. This damage is caused directly through larval feeding of *Lobesia botrana*, and indirectly by *Scaphoideus titanus* as a vector of a dangerous quarantine Phytoplasma disease (Flavescence dorée). Consequently, they are the target of different pest management strategies. They are mainly controlled with the use of pesticides and mating disruption for *L. botrana* only. The raising awareness on the impact of pesticides on the human health and the environment, concerns about pesticide resistance, the spread of these species into new regions and the recent challenges such as climate change all underline the necessity of more efficient control for those pests based on an improved knowledge of their biology and ecology.

A more comprehensive understanding of the insect phenology and behaviour could be the first step in building a coherent and successful control strategy. This is the main motivation to conduct this research. In this thesis the phenology (I) and the genetic structure of populations (II) of *L. botrana* and regarding *S. titanus* the phenology (III), behavior (IV) and Grapevine Phytoplasmas interaction (V) were studied

(I) the phenology of grape berry moths *Lobesia botrana* and *Eupoecilia ambiguella* was investigated in several sites in Veneto region during the period of 2010 and 2011. Few sites were also studied in 2012. The results show a clear preference for *E. ambiguella* to hilly areas and for *L. botrana* to plain areas. Number of generations per year varied among the sites, and the beginning of the flights were generally earlier in 2011. For *E. ambiguella* the observed generation numbers was 2 to 3. Regarding *L. botrana* 2 to 4 flights were recorded. In some areas *L. botrana* showed a long flight period which made it difficult to determine whether it was one or two overlapping flights. However, this phenomenon could be associated with larval aestivation. Moreover, four flights were noticed for *Lobesia botrana* in some areas, but no evidence for fourth generation was provided. Temperature was found to have an influence on the second flight of *E. ambiguella* and the beginning of this flight for *L. botrana*.

(II) The genetic structure of populations of *L. botrana* was studied, because, in spite of the importance of grape berry moth, little is known about its genetic structure. In this study, six microsatellite loci were used to analyse 16 population of *Lobesia botrana* from the Middle East and

Europe, to investigate their variation and structure. The results showed moderate levels of genetic differentiation among the different populations. An excess heterozygosity was noticed in most of the populations due to the mating among the populations. The populations coming from Middle East and Spain belong to different genetic clusters, while the German and Italian ones were mixed. No clear geographical effect was detected on the different studied populations. These data are relevant for the development of pest control strategies because, the study of the gene flow of populations could help in knowing the distance over which the population could disperse, consequently, knowing the spatial scale within which the forecasting techniques could be effective.

(III) The phenology of *S. titanus* was studied in several sites in Veneto region in 2010-2012 in order to know the best time to apply the different pest management practices. The collected data showed that the *S. titanus* nymphs appear in May, while the adults appeared, between the third part of June and the mid of July, and disappeared between September and early October. In general, *S. titanus* nymphs and adults appeared earlier in 2011 and 2012 than in 2010 with some variations among the sites depending on altitude and sun exposure. The temperature affects the nymphs phenology and the nymphs appearance and development are synchronized with the host phenology, while for the adults there are other factors that could affect the phenology beside the temperature such as altitude and photoperiod.

(IV) The study on the preference of *Scaphoideus titanus* to different grape cultivars, and the role of stimuli in host recognition was conducted because these aspects are not well known and explained for this leafhopper. In this study four grapevine Cultivars were used (Chardonnay, Cabernet franc, Merlot and Glera) to study their attractiveness to different life stages of *S. titanus*. Furthermore, healthy and infected shoots by Grape Yellow Phytoplasma Diseases (Flavescence dorée and Bois noir) were used to investigate the ability of the leafhopper to distinguish among their different stimuli. The results of this study showed that there are different levels of preference rates for *S. titanus* towards the investigated cultivars. This preference varied according to the age and throughout the experiment period. Chardonnay was more attractive to the young nymphs and nymphs. Young nymphs and adults seemed to prefer Cabernet Franc. Moreover, *S. titanus* was found to be able to distinguish among healthy and GYs infected stimuli, while no strong attraction was detected of this insect to the stimuli of FD, BN phytoplasma. These results could provide help in understanding the insect behaviour and distribution within the vineyard. Hence, they could contribute to the development of stronger tools for the monitoring and control strategy.

(V) The effect of the Grapevine Yellow Phytoplasmas on the development and the survival of *Scaphoideus titanus* was studied using grapevine leaves as phytoplasma infection source. In

addition to Flavescence dorée (FD) the Bois noir (BN) causal agent was also investigated because its importance and occurrence are increasing in many Italian regions, and because it was detected inside *Scaphoideus titanus*. Although this leafhopper is not involved in BN transmission and spread, the BN phytoplasmas could have an effect on the insect life. Newly hatched nymphs were collected on asymptomatic shoots and reared artificially on healthy, FD or BN infected leaves, under controlled conditions. The results showed that nymphs reared on phytoplasma infected leaves had a greatly longer longevity and lower survival rate, while no differences between FD and BN were found. The phytoplasmas detection analyses confirmed the ability of S. titanus in acquiring the causal agent of BN.

Chapter 1

Introduction

Grapevine: Importance and Challenges

The grapevine (*Vitis vinifera* L.) is a fruit crop widely distributed in the world (Vivier and Pretorius, 2002). It includes the wild grapevine (*Vitis vinifera* spp. *Silvestris*) which is considered to be the origin of the domesticated grapevine *V. vinifera* spp. *sativa* (De Mattia *et al*., 2008). The first evidence of grape cultivation came from the Middle East (Zohary and Hopf, 2000). Grapevine is a water-stress adapted crop (Flexas *et al*., 1998), hence it could be easily spread to new environments such as the tropical and semi-tropical (Camargo *et al.*, 2007). Moreover, grape has a very good economic value (Arroyo-Garcia *et al.*, 2006) and it could be consumed as fresh fruit, wine, juice and leaves due to its high nutritional content (Didem *et al.*, 2009).

Nowadays there are many challenges that the grapevine growers have to face including climate change, invasive pests, developing of more efficient agronomic practices and plant-protection strategies. They could be considered as major factors potentially involved in the evolution of arthropods associated problems in viticulture.

Climate change is the most important challenge to viticulture worldwide, in particular to the wine industry (Jones *et al.*, 2005). Climate has a big contribution to grapevine growth and to the quality of wine (Jones and Davis, 2000). On the other hand, climate change could influence the presence of pests and diseases (Jones *et al.*, 2005), more specifically, it can induce an expansion of the geographic range of grape leafhoppers and berry moths. Moreover, climate change can alter pest phenology, determining an increase in the number of generations per year.

Invasive pest occurrences have complicated the plant-protection management in some areas. The impact of horticultural techniques on grapevine arthropods community is increasing in importance, interesting examples are provided by the effect of the weed management on Bois Noir BN spread, and the interactions between water management and *Empoasca vitis*.

However, the plant protection techniques depend largely on a combination of pesticides use, agronomical practices (pruning, variety selection, soil tillage…) and biological control methods. As a consequence of the damage pesticides may cause to the human and environmental health, the public demand for producing food free from chemical residuals, and the non-efficiency of the available alternatives, other agricultural production systems have been developed as organic agriculture.

Grapevine was among the first crops which were managed organically (Geier, 2006), since 1950s (Willer, 2008), because of the importance of this crop. In 2010, the organic viticulture area was more than 200.000 ha, which is approximately 2.9% of the world's total grape area (FAO, 2010). In Italy, the statistics show an increase of about 20% in the organic grape area between 2009 and 2010 (Willer and Kilcher, 2012).

Pests and diseases are a constant risk to grape production and quality, this risk is even greater for the organic growers who are not allowed to use chemical pesticides (Magarey *et al*., 2000). In spite of the availability of some organic plant protection techniques such as, agronomical practices and natural insecticides, these techniques are not always effective and they are highly influenced by the climatic conditions when applied in the field (Handelsman, 2002).

There are several pests that can infect grapevine. The most relevant one is grape berry moth (*Lobesia botrana* Schiff) which is widely distributed in the world. Whereas the grapevine leaf hopper (*Scaphoideus titanus* Ball) does not cause a direct damage to grape, but it is a vector of a quarantine phytoplasma disease in Europe (Flavescence Dorée) which makes it another important pest for viticulture.

Grape Berry Moth (*Lobesia botrana* Schiff)

The grape berry moth *Lobesia botrana (*Denis & Schiffermüller) (Lepidoptera: Tortricidae) is a major pest in all grape growing areas.

L. botrana was firstly described by Denis and Schiffermüller in Austria in 1776 (Maher and Thiéry, 2006). It is thought to be a key pest on grape since the Roman times (Kreiter, 2000). Nowadays, *L. botrana* is present throughout the Palaearctic region (Bovey, 1966). It is found in Middle Europe, the Mediterranean countries, southern Russia, Japan, the Middle East, and northern and western Africa (Venette *et al*., 2003; Thiéry and Moreau, 2005; Maher and Thiéry, 2006) and recently in California in USA (Varela *et al*, 2010; Todd *et al.*, 2011; Gutierrez *et al.*, 2012).

Grape berry moth is polyphagous insect which occur and complete its development on more than 20 species (Bovey, 1966; Stoeva, 1982; Thiéry, 2005; Thiéry and Moreau, 2005). Maher and Thiéry (2006) found out that the ever green shrub (*Daphne gnidium* L.), which grows wildly in south Europe and the Mediterranean basin, can stimulate the oviposition of *L. botrana*. This supports the hypothesis that says *L. botrana* shifted to grape from other wild plants, and that *D.*

gnidium could be the ancestral host plant for grape berry moth (Balachowsky and Mesnil, 1935; Bovey, 1966; Stoeva, 1982; Thiéry and Moreau, 2005). In the same context, there are some reports that show the presence of *L. botrana* larvae on olive trees in north Greece and Bulgaria (Tzanakakis and Savopoulou, 1973; Stoeva, 1982). Those alternative hosts (Daphne and olive) were found to increase *Lobesia botrana* fitness even more than grape (Thiéry and Moreau, 2005). However, grape continues to be the main food resource for grape berry moth (Maher and Thiéry, 2006).

This moth has more than one generation per year, the number of generations depends on the temperature, photoperiod (Martìn-Vertedor *et al.*, 2010) and the latitude (Roehrich and Boller, 1991) and each generation lasts from 1-2 months depending on the region (Moreau *et al.*, 2010). In central Europe (Austria, Germany, Switzerland and central France) *L. botrana* completes two generations per year. While in southern Europe (south France, south Italy, Spain, Portugal, Greece), the rest of the Mediterranean countries such as (Algeria, Tunisia and Syria) and in Iran, it has three to four generations (Bovey, 1966; Coscollá, 1997; Badenhausser *et al.*, 1999; Ibrahim and Al-Radwan, 2006; Bounaceur *et al*., 2011; Akbarzadeh Shoukat, 2012).

The female of *Lobesia botrana* oviposits on the floral buds and berries from spring to autumn (Maher and Thiéry, 2006). Number of eggs varies between 70-150 according to climatic conditions and to the quality of food and its abundance to larvae (Maher and Thiéry, 2006). The females are able to distinguish among different grape cultivars (Maher *et al*, 2001; Moreau *et al.*, 2008). However, females tend to prefer the host species on which they developed as larvae (Moreau *et al*, 2008), but they also select the plant species which will increase the larval growth and survival (Courtney and Kibota, 1990; Thompson and Pellmyr, 1991; Leather, 1994; Janz, 2002). The egg size is controlled by physiological and environmental factors (e.g. female age at mating, availability of water, larval feeding and pupal weight). On the other hand, a bigger egg size could significantly enhance larval performance (Torres-Vila and Rodríguez-Molina, 2002).

The eggs hatch and give larvae that develop in five instars (Bovey, 1966). The width of head capsule could be used to distinguish among the different instars (Irigaray *et al.*, 2006; Delbac *et al.*, 2010). The total development time for larvae depends on the plant species (Thiéry and Moreau, 2005), as what was reported in Greece (Savopoulou-Soultani *et al.,* 1990) where on olive the development time was two to three days faster than on grape. Likewise, the larval food could have a big impact on the mating ability of *L. botrana* adults (Moreau *et al.*, 2007). In

addition to the plant species and grape cultivar, the grape phenological stage during the larval stage could also affect the reproduction of adults and their weight (Torres-Vila, 1996; Torres-Vila *et al*., 1999). The first generation larvae feeds on grape flowers giving a low adult weight and mating ability, while the second and third generations feed on berries and ripe berries respectively, resulting on a higher adults' weight and reproduction performance (Badenhausser *et al.*, 1999; Pavan *et al.*, 2010). At the end of larval stage, larvae convert into pupae.

Lobesia botrana overwinters as diapausing pupae, the length of the photoperiod is the main factor which affects the diapauses of *L. botrana* pupae (Pavan *et al.*, 2010). In that case, a short photoperiod leads to an increase in the development period of the larvae, causing the pupae to be heavier (Deseö *et al*. 1981; Roditakis and Karandinos, 2001). Figure 1 demonstrates the different life stages of *Lobesia botrana*.

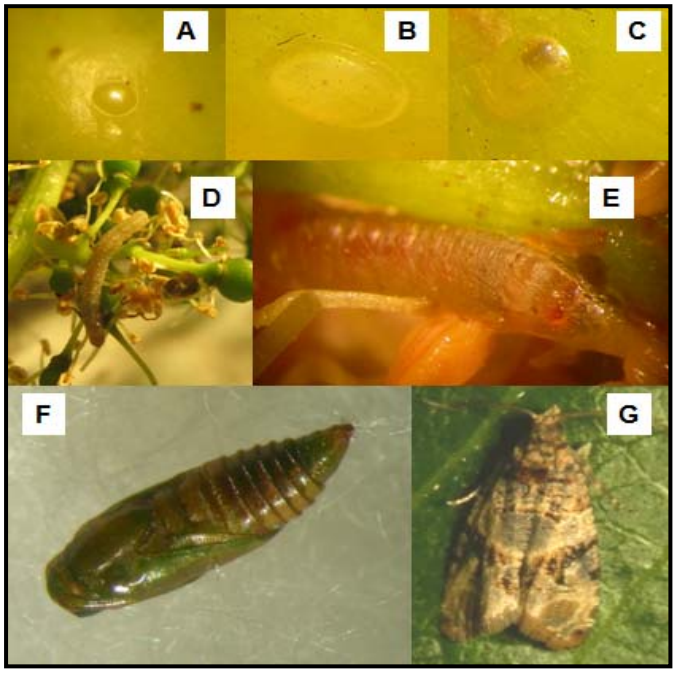

Figure 1- Different life stages of *Lobesia botrana*. (A, B and C) represent the three instars of *L. botrana* egg. (D) the larva. (E) larva becoming a pupa. (F) the pupa and (G) the adult

Figure 2 shows the life cycle of grape berry moth (*Lobesia botrana*) in North Italy (Zangheri *et al*., 1992).

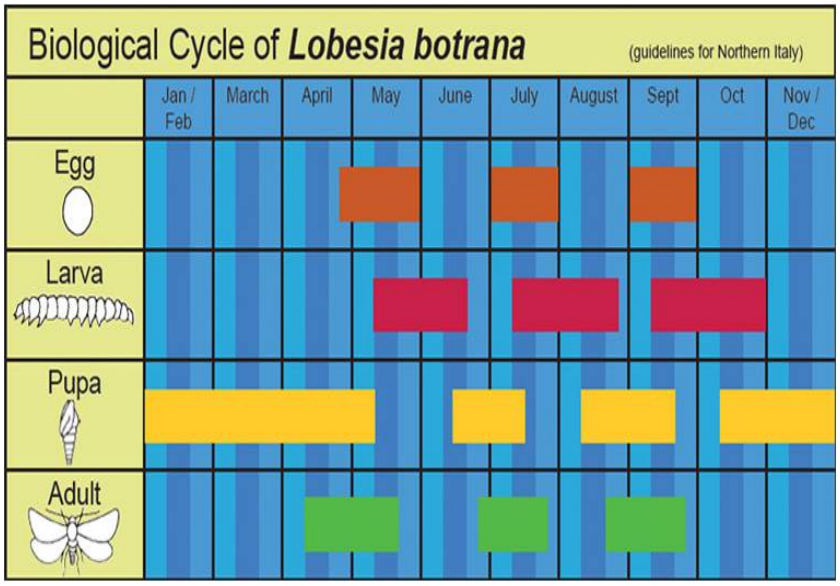

Figure 2- *Lobesia botrana* life cycle in north Italy (Zangheri *et al*., 1992)

Grape berry moth (*Lobesia botrana*) has a very big economic importance due to the damage it makes to both the quality and the yield of grape (Bovey, 1966). Larvae are considered to be the most damaging life stage of *L. botrana* because of their feeding activity (Caffara *et al.*, 2012). For the first generation of grape berry moth, the newly hatched larvae feed on the flower buds, which causes kind of thinning to the clusters (Zahavi *et al.,* 2003), and later on, they gather several buds together by making glomerules. The glomerules provide protection to the larvae while they continue their feeding. The tolerance level of the first generation is relatively high, 15 to 100 larvae per 100 grape flowers, corresponding to approximately 60% of bunches attached (Pavan and Girolami, 1986; Roehrich and Boller, 1991), and it seems to depend on the grape variety ability to compensate the damage (Roehrich and Schmid, 1979; Badenhausser *et al*., 1999). The second and the third generations are the most damaging. The larvae of these generations feed on berries and mature berries causing a direct damage represented by the loss in the production weight in particularly with the 2nd generation (Pavan *et al*., 1998). In addition to the production quantity damage, there is a reduction in the quality especially for table grape varieties where the accepted level of damage is zero (Reynaud, 2003). As a consequence of the larval feeding, an indirect damage could occur, by facilitating the infection with fungal diseases (e.g. *Botrytis cinerea*) (Deseö *et al*., 1981; Badenhausser *et al*., 1999; Zahavi *et al*., 2003) during the season, or the black aspergilla rot (*Aspergilus niger* and *Aspergilus carbonarius*) (Cozzi *et al.*, 2006). The damage of *Lobesia botrana* depends from one side on how much the clusters are

compact (Pavan *et al*., 1993), and on the other side on the phenological stage of grape (Roerich and Boller, 1991). The damage caused by the larval feeding on grape is presented in figure 3.

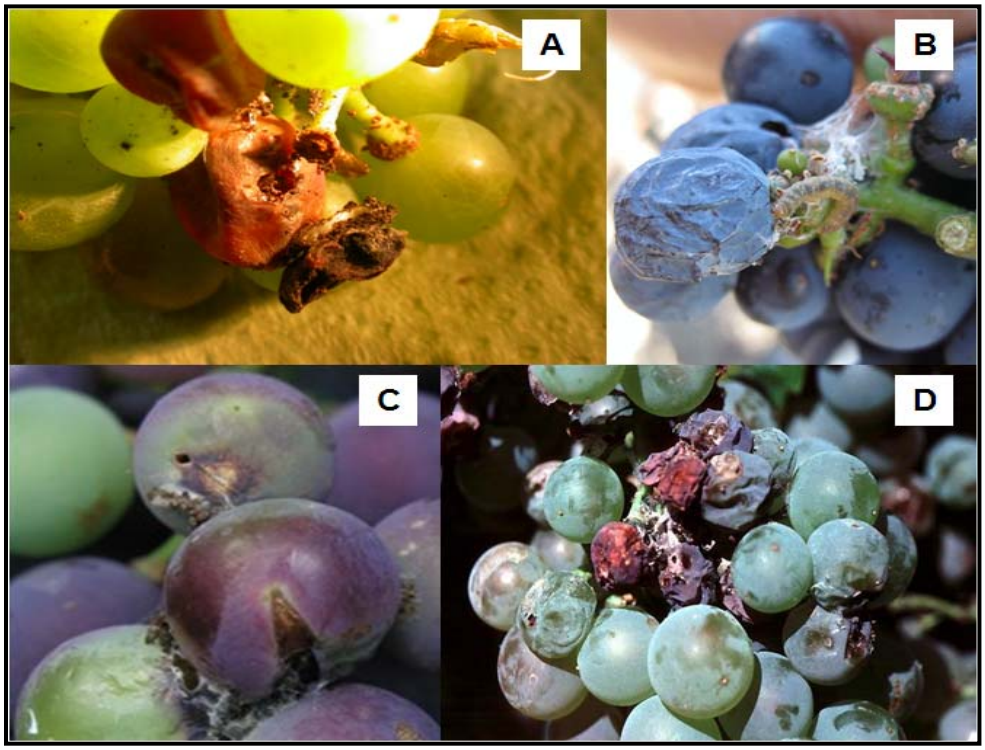

Figure 3- (A) hollowed berries after *L. botrana* larval feeding. (B) Larva is feeding on berries (photo by Vasquez S.J., 2009). (C) Larva penetrates the berries thus could facilitate fungal diseases (photo by Rigs N., 2012). (D) The damage on grape bunches (photo by Del Estal P., 2007).

As a result of this economical importance, the different pest management programs considered *Lobesia botrana* as a main target (Moreau *et al.*, 2010). The most traditional method to control pests and diseases are the pesticides, which were used extensively against *L. botrana* (Irigaray *et al.*, 2006; Moreau *et al.*, 2010). The most common compounds recently are insect growth regulators (IGRs) (Hosseinzadeh *et al*., 2011), chitin synthesis inhibitors (e.g. flufenoxuron) and moulting accelerating compounds (e.g. tebufenozide) (Charmillot, 1989; Charmillot *et al.,* 1994; Pavan *et al.*, 2005a). Selective pesticides are also used (i.e., benzoyl phenil ureas BPUs) but their efficiency depends a lot on knowing precisely the development stage of the insect (Irigaray *et al.*, 2006). Currently, the use of ovicides before and during the ovipositing period is to be thought an efficient method for grape protection against *L. botrana*. However, the oviposition period is difficult to determine, and this method could need a direct contact with the eggs (Delbac *et al.*, 2010). But, the increasing cost of applying pesticides, the risk of resistant development (Ioriatti

et al., 2002), the increasing aware on the damage the pesticides cause to the environment and human health and the side effect they could cause (for example, when controlling the first generation of *L. botrana* an outbreak of spider mites could be noticed at the same time, reported in Duso *et al*., (1989)), lead to the need to develop other alternative techniques to control grape berry moth. A good alternative for pesticides is mating disruption, it has been widely used in viticulture against *L. botrana* since 1994 (Louis *et al*., 2002). This technique depends on the emission of female pheromone which is naturally used by the female moth to attract males for mating. This leads to male disorientation and the reduction of pest offspring thus the damage (Varner *et al*., 2001; Mazzocchetti *et al*., 2004; Ioriatti *et al*., 2004; Bagnoli *et al*., 2006; Marchesini *et al*., 2006; Lucchi *et al.,* 2007; Bigot *et al*., 2008; Duso *et al*., 2010a). The only problem of this method is that it requires low density of the pest (Feldhege *et al*., 1993; Varner *et al*., 2001). Moreover, another promising alternative for the control of *L. botrana* is biological control which depends on the use of beneficial living organisms (Gurr and Wratten, 2000; Eilenberg *et al*., 2001). One of the most famous biological control agent is *Bacillus thuringiensis,* in fact, *L. botrana* was successfully controlled using this bacterium particularly with the strains (3 and 34) (Ruiz de Escudero *et al*., 2007). A more successful biological control strategy is to favor the use of already existing natural enemies, this requires scanning to identify the possible natural enemies in a given environment and the knowledge on the interaction between the pest, the host and the natural enemy (Van Lenteren, 2006; Moreua *et al*., 2010; Duso *et al*., 2010a). There are several studies that provide an adequate knowledge about the presence of natural enemies in some regions, among these studies (Marchesini and Dalla Montà, 1994; Moreau *et al*., 2010) and they proved the ability of the parasitoids to control *Lobesia botrana*. Most of the parasitoids belong to the families Hymenoptera or Diptera (Thiéry *et al*., 2001; Chuche *et al*., 2006). As an example of these parasitoids, the wasp *Dibrachys cavus,* was found on overwintering pupae of *Lobesia botrana* in several European countries including Italy (Marchesini and Dallà Monta, 1994; Chuche *et al*., 2006). However, the parasitoids species of *L. botrana* differ from a region to the other, and they are affected by the grape cultivar. Nevertheless, the interaction among the pest and its surrounding should be taken into account when using biological control programs (Moreau *et al*., 2010).

Grape leafhopper *Scaphoideus titanus* Ball

The grape leafhopper *Scaphoideus titanus* (Homoptera: Cicadellidae) is a dangerous pest for viticulture, because it is a vector of 16SrV *Candidatus* Phytoplasma vitis, the causal agent of the Grape Yellow Disease Flavescence Dorée (FD). (Schvester *et al*., 1961; Carraro *et al*., 1994; Bianco *et al*., 2001; Mori *et al*., 2002).

This Nearctic leafhopper has an American origin of the Great lake region (Vidano, 1966), it was introduced to Europe between 1950-1960 through the south of France (Bonfils and Schvester, 1960; Vidano, 1966; Boudon-Padieu, 2000a) by importing grapevine canes with eggs under the bark (Caudwell, 1983). A recent study by Papura *et al*. (2012) used a combination of nuclear and mitochondrial markers to trace back the invasion history of *S. titanus* to Europe Confirmed the American origin of this leafhopper, and suggested that the introduction of this pest happened once or several times from the same area. Nowadays, *S. titanus* could be found more in the eastern and northern vineyards of Europe more than in the southern Mediterranean parts (Boudon-Padieu, 2000a; Chuche and Thiéry, 2009). *S. titanus* is spread in France, Italy, Spain, Switzerland, Serbia, Croatia, Slovenia, Austria, Hungary and Portugal (Bonfils and Schvester, 1960; Vidano, 1964; Quartau *et al*., 2001; DeSousa *et al*., 2003; Alma, 2004; Magud and Toševski, 2004; Mazzoni *et al*., 2005; Lessio and Alma, 2006; Steffek *et al*., 2007; Dér *et al*., 2007; Gabrijel, 2008). In Italy, *S. titanus* was detected for the first time by Prof. Carlo Vidano in 1964 in Liguria. Now, it is found in the northern and central Italian regions (Sancassani *et al*., 2008) and also under the latitudes 40° N in Basilicata and Campagna regions (Viggiani *et al*., 2002; 2004).

In its native area *S. titanus* could be found on different grasses, shrubs and trees (Gibson, 1973; Barnett, 1976; Hill and Sinclair, 2000; Mazzoni *et al*., 2009), as well as, on different grape species such as the wild *Vitis riparia* and the cultivated *Vitis vinifera*, but with more preference to the wild species *V. riparia* (Maixner *et al*., 1993; Beanland *et al*., 2006). Although in Europe *Scaphoideus titanus* is considered a monophagous insect which feeds only on grape vine (Vidano, 1966; Lessio and Alma, 2006; Decante and Helden, 2006; Lessio *et al*., 2007), it might feed on other plants in the laboratory (*Cineraria maritime* and *Vicia faba*) and in nature (*Ulmus americana*) (Caudwell *et al*., 1970; Gibson, 1973).

Scaphoideus titanus has one generation per year (Vidano, 1966; Pavan *et al*., 1987) and it is a hemimetabolous phloem-feeding insect (Conti and Vidano, 1988; Bertin *et al*., 2007). The female oviposits on the woody canes from summer till autumn, the node area was more favorable for the females to lay the eggs, according to some tests (Bagnoli and Gargani, 2011). However, some studies demonstrate that the females of *S. titanus* could also lay the eggs on one year old wood, although the hatching rate would be lower than the two year old wood by 20-30 times (Forte *et al*., 2010). Eggs are the overwintering stage of *S. titanus* (Vidano, 1966), and they hibernate under a two year old wood bark. Nymphs hatch from the eggs from May of the following year (Boudon-Padieu, 2000a; b). The nymphs manage to survive if the hatching took place with or shortly after grapevine bud bursting, when the leaves are young and they have high nitrogen content (Mooney and Gulmon, 1982). They could be found on the lower side of grapevine leaves, and they do not go for big distance. They feed by sucking the sap from the small venations, whereas the older nymphs (4^{th} and 5^{th} instars) could feed on the stems and the green shoots (Vidano, 1966). Nymphs occur in spring from May till the end of June in Italy (Pavan *et al*., 1987; Lessio and Alma, 2006) or till July in France (Boudon-Padieu, 2000b). Nymphs develop through five instars (Vidano, 1966) with different dimensions and colors that facilitate the ability to distinguish among them (Dal Ri and Capra, 2003). Some studies state that, starting from the third instar nymphs the acquisition of phytoplasma could begin, this is due to the feeding behavior of the 1^{st} and 2^{nd} instar nymphs (Carle and Moutous, 1965). While for other studies, all the stages of *S. titanus* may acquire the phytoplasma (Bertin *et al*., 2007) but the possibility to detect the phytoplasma acquisition increases with the insect age (Bressan *et al*., 2006). The acquisition begins through the feeding process on already infected plants (Lessio and Alma, 2006), which appears to be the only source for the vector natural acquisition (Bressan *et al*., 2006). The acquisition efficiency is affected by several factors such as the susceptibility of the grape cultivar, the nymph instar and the growing season (Bressan *et al*., 2005b). The transovarial does not seem to be the way to transmit the phytoplasma through the different generations (Bressan *et al*., 2005a). During the feeding the insect inoculates the phytoplasma into the phloem of healthy plants (Pajoro *et al*., 2008), so the vector spread the disease from vine-to-vine which leads to the expansion of the disease over the years (Van der Plank, 1963; Pavan *et al*., 1997). Then, after a latency period of 28-35 days, the adults could infect new plants (Schvester *et al*., 1969). During the latency period, the phytoplasma can multiply and persist inside the vector's body (Conti and Vidano, 1988; Bressan *et al*., 2006). The adults are

considered as the most important life stage in the spreading of this disease (Bressan *et al*., 2006). Adults are found in mid-summer from mid July to mid October (Pavan *et al*., 1987) their flight is mainly concentrated between the evening and the early morning, other than that, they do not tend to move far from the plant (Lessio and Alma, 2004a; b). Consequently, it is thought that human trading activities may have the essential role in the long distance dispersal of this insect (Bertin *et al*., 2007).The adults distribution in the field is noticed to be highly varied (Duso *et al*., 2010b) and could be influenced by the planting system (Lessio and Alma, 2004a; 2006). The adults and nymphs show aggregated pattern of distribution, but this social behavior could be because of the attraction to the plant or to the group (Bosco *et al*., 1997; Lessio and Alma, 2006). Figure 4 presents the different life stages of *S. titanus*.

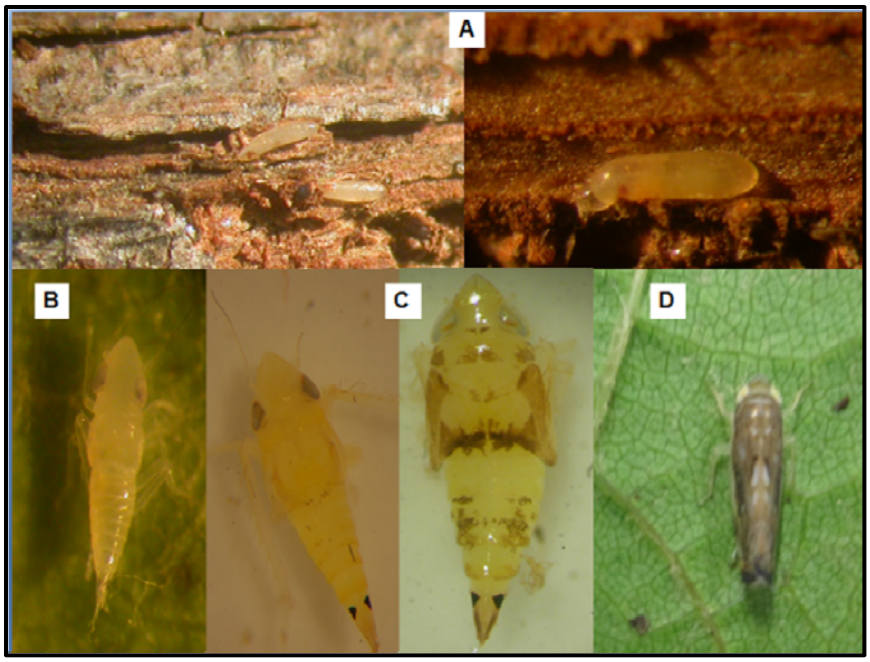

Figure 4- Different life stages of *Scaphoideus titanus*. A: the eggs B: young nymphs C: old nymphs and D: the adult

The economic importance of *Scaphoideus titanus* does not come from the direct feeding on the grapevine leaves (Mazzoni *et al*., 2009) but from being a vector of the *Candidatus* Phytoplasma vitis, Flavescence dorée FD disease which is a main threat to viticulture and is considered as a quarantine infectious disease in Europe because of its big damage, and due to its rapid spread by its specific vector *S. titanus* (Schvester *et al*., 1961; Boudon-Padieu, 2003).

As a consequence of the importance of FD disease chemical treatment is compulsory (Planas, 1987; Barba, 2005; Pavan *et al*., 2005b). Since, there is no direct control to the casual agent of

Flavescence dorée (Marzorati *et al*., 2006), so all the control strategies are targeting the vector *S. titanus*. The control includes in addition to the insecticide treatments the rouging of the infected plants (Weintraub and Beanland, 2006). It could be also necessary to eradicate the abandoned neighbouring vineyards and the American grapevines growing wild in spontaneous vegetation (Pavan *et al*. 2012), because they could serve as a reservoir for *S. titanus* (Lessio *et al*., 2007; Pavan *et al*., 2012). Regarding the number of pesticides treatments, there are usually one to three sprays per year depending on the FD presence (Pavan *et al*., 2005b) and on the insect population density. The number of treatments against *S. titanus* varies according to the country. For example, in Switzerland, insect growth regulators are used twice per year, but they require an accurate knowledge on the phenology and the appearance dates of the youngs and adults (Rigamonti *et al*., 2011). While in Italy where FD is present, at least one treatment is compulsory, and the used compounds were chitin depressors and neurotoxic products (Bosio *et al*., 2004; Pavan *et al*., 2005b) and, nowadays, neonicotinoidis. The first treatment is in June against the nymphs, while the second treatment (if needed) is in July (Pavan *et al*., 2005b). In organic agriculture pyrethrum could be applied (Caobelli and Carcereri, 1995; Caruso and Mazio, 2004), but due to the high sensitivity of the active ingredients which are allowed in organic agriculture to temperature and light, treatments should be made with high application volume and preferably in the evening (Bottura *et al*; 2003).

However, the efficient control depends on the availability of a correct monitoring of *S. titanus* (Posenato *et al*., 2001; Lessio *et al*., 2011).

In agreement with the recent European directive 128/2009, that requires a sustainable use of pesticides, it is very important to reduce the use of pesticides and apply more environmentally friendly methods. Some of these alternatives could be trying to disturb the mating process by pheromone dispensers, till now there are not studies about the role of chemical communications in leafhoppers. Instead, the vibration signals is the method used by *S. titanus* for mating, and Mazzoni *et al.* (2009) found out that playing back some disruptive vibration signals could interrupt the female-male duet and manage the population of this leafhopper. It could be useful for a better control of the insect, to use preventive measures such as the right selection of grape cultivar, Bressan *et al*. (2005c) showed that the resistant cultivars to FD are a bad acquisition source for *S. titanus*. The use of disease-free propagating material could be also a possible way to prevent the infection in the field (Caudwell *et al*., 1997). The propagating material may carry the

overwintering eggs of *Scaphoideus titanus*, the fact that made Caudwell *et al*. (1997) conduct through the years several experiments on the elimination of those eggs with the use of hot water (of 50°C for 45 minutes). In spite of the increasing need for biological control agents, there are few studies in this regard. An interesting field for the ecologically control of *S. titanus* is the "symbiotic control" that is based on the use of the microorganisms symbionts in the host body, to interfere with the FD pathogen itself (Bextine *et al*., 2004). In that context, a study was done by (Marzorati *et al*., 2006) and found out that there are two bacteria colonizing the body of *S. titanus* they could be used, after further studies, for the symbiotic control of the FD agent.

Research motivation

Based on what was stated before:

- The big importance of grapevine especially for Europe in general, and Italy in particular as a big producer of grape fruit and wine;
- The economical importance of both insects and the damage they cause to the quality and the quantity of grapevine production;
- Changing climate, the spread of the previously mentioned pests into new regions;
- The negative impact of pesticides on the environment and human health, and the ban on the use of chemicals in other agricultural production systems such as organic agriculture;
- The need to develop an efficient ecological method to control these pests.

All these factors raise the question of how to control better *Lobesia botrana* and *Scaphoideus titanus*.

A more comprehensive understanding of the insect phenology and behavior could be the first step in building a coherent and successful control strategy. This is the main motivation to conduct this research.

The objectives

The present research deals with the following objectives:

1. Study the phenology of both insects in several locations in Veneto (north-eastern Italy). The phenological data are going to be used to develop and/or improve forecasting models which will provide the farmers and the technicians with reliable information for the best timing of the pest treatment.
2. Study the population genetic structure of several *L. botrana* populations from Middle East and Europe with the use of microsatellite loci. The purpose of this study is to shed the light on the population dynamic and structure of grape berry moth, which could help for developing rational control strategies.
3. The attraction of *Scaphoideus titanus* towards grapevine varieties and their response to stimuli emitted by healthy or infected grapevine leaves.

4. The effect of phytoplasma on the longevity and the survival of *S. titanus*. Since, most of the studies are focusing more on Flavescence dorée than its vector, these studies could help to provide a boarder view on the vector itself, in order to improve the used strategy against the leafhopper.

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Chapter 2

The phenology of grape berry moths *Eupoecilia ambiguella* and *Lobesia botrana* (Lepidoptera: Cochylidae, Tortricidae) in Veneto region (North-eastern Italy)

Haya AbouAssaf collected part of the data, contributed to their analysis and drafted the manuscript. This work was made in cooperation with Dr. Alberto Pozzebon, Dr. Diego Fornasiero, Dr. Mauro Lorenzon, Dr. Nicola Mori and Prof. Carlo Duso.

Abstract

The grape berry moths *Eupoecilia ambiguella* and *Lobesia botrana* are the most important grapevine pests in European vineyards. Although their distribution is different in grapevine growing areas they can co-exist in many regions as in North-eastern Italy. The phenology of grape berry moths was investigated in several sites in Veneto region in 2010 and 2011. Few sites were also included in 2012. *E. ambiguella* was more common in hilly areas while *L. botrana* in plain areas. The number of flights per year varied among the sites and the beginning of flights were generally earlier in 2011. *Lobesia botrana* showed in some areas a long flight period which made it difficult to determine whether it was one or two overlapping flights. This phenomenon could be associated with larval aestivation. Four flights were noticed for *L. botrana* in some areas, but no evidence for four larval generations was provided. Temperature had an effect on the second flight of both berry moths.

Introduction

The grape berry moths *Lobesia botrana* (Denis & Schiffermüller) and *Eupoecilia ambiguella* (Hübner) are major pests in European viticulture because of the considerable damage they cause to the quality and quantity of grape production (e.g., Bovey, 1966; Kast, 1990). This damage is directly caused by feeding on the flower clusters and grape berries and indirectly by facilitating the infection of pathogens, mainly *Botrytis cinerea* (Deseö *et al*., 1981; Mondy *et al.,* 1998; Badenhausser *et al*., 1999; Zahavi *et al*., 2003; Dalla Montà *et al.,* 2007) and increasing the risk of ochratoxin contamination of grape berries (Cozzi *et al.,* 2006; Visconti *et al.*, 2008). These polyphagous species have different ecological requirements (Bovey, 1966; Galet, 1982) which are reflected by their distribution throughout the European grapevine areas. *Lobesia botrana* is found to be more dominant in warmer regions of central and south Europe, while *E. ambiguella* seems to prefer northern European regions (Bovey, 1966; Chalverat, 1978; Galet, 1982; Zangheri *et al*., 1987; Sobreiro, 1989; Zangheri and Dalla Montà, 1989). However, *L. botrana* and *E. ambiguella* coexist in some regions as in north-eastern Italy (Pavan *et al*., 1994). In these transition areas, climatic conditions could influence the dominance of one species over the other. Warmer years favour the presence of *L. botrana*, while rainy and cold years help the

development of *E. ambiguella* (Schirra and Louis, 2001; Bărbuceanu, 2005). Both species are able to develop more than one generation per year depending on climatic conditions (Martìn-Vertedor *et al.*, 2010). In north-eastern Italy, *E. ambiguella* develops two generations per year, but a third generation is also commonly observed; *L. botrana* has usually three generations per year but in hot seasons a fourth generation was noticed in north-eastern Italy (Zangheri and Dalla Montà, 1989; Pavan *et al*., 1994; Marchesini and Dalla Montà, 2004; Pavan *et al*., 2006).

Grape berry moths could be controlled by pesticides or other alternatives as mating disruption (Charmillot *et al*., 1995; Schirra and Louis, 2001; Schmidt-Büsser *et al*., 2009; Moreau *et al.*, 2010) or by a combination of both of them since pheromone traps were found to be less effective with high infestation rates of grape berry moths (Feldhege *et al*., 1993; Varner *et al*., 2001). The use of pesticides in agricultural production should be reduced according to European rules (Dir. 2009/128/CE). Therefore it is crucial to identify the best timing of treatment and environmental friendly control methods. In any case, the key for successful pest management program is a comprehensive knowledge of the pest phenology (Patrick *et al*., 2003). The phenological data is considered as the fundamental part in building day-degrees models which are used to predict the development progress of the insect linked with the climatic conditions, to provide decision support systems for efficient insecticide application timing (Riedl *et al*., 1976; Touzeau, 1981).

The objective of this study was to investigate the phenology of *L. botrana* and *E. ambiguella* in some sites of Veneto region (north-eastern Italy) along an altitude gradient (Table 1).

Materials and methods

Experimental sites

Seven sites were selected to study the phenology of both grape berry moths in Veneto region (Table 1). These fields have different histories and cropping systems but no insecticides were applied during the studying period.

Insect sampling

The adults of both species were captured using pheromone traps (Isagro Traptest®) to study the beginning of each flight, its duration and variation among the years and the sites. The study lasted for two years (2010-2011). Nevertheless, Valdobbiadene 2, Roncade and Meolo were also

considered in 2012. In some sites traps were located after the beginning of the first flight that is poorly considered in this work.

Table 1- Sampling locations in Veneto region

n.	Location	Province	GPS cod.	Altitude (m.a.s.l.)
1	Feltre	Belluno	46°1′0″N 11°54′0″E	540
2	Fonzaso	Belluno	46°1′0″N 11°48′0″E	329
3	Valdobbiadene 1	Treviso	45°53′46″N 11°57′54″E	240
4	Valdobbiadene 2	Treviso	45° 54′ 0″ N, 11° 55′ 0″ E	105
5	Pernumia	Padova	45°16′0″N 11°47′0″E	9
6	Roncade	Treviso	45°37′41″N 12°22′30″E	8
7	Meolo	Venezia	45° 37′ 13.08″ N, 12° 27′ 21.24″ E	2

Climatic data

The minimum, medium and maximum temperatures were provided by ARPAV (Agenzia Regionale per la Prevenzione e la Protezione Ambientale del Veneto), from the nearest weather station to each site. The greatest distance between the investigated vineyards and the weather stations was 3.6 km. Regression analyses were performed by R software release 2.15.0 (R Development Core Team, 2011), to study the relation between average medium and maximum temperatures and the dates of second flight beginning for both moths or the date of 50% of adult captures. Those dates were expressed by Julian days. Temperatures values used here are the averages of medium and maximum temperatures in the month when flight occurred and in two months before (e.g., if the flight started in June, average temperatures of April+May+June were used).

Results

Phenology of grape berry moths

Eupoecilia ambiguella

At Feltre, traps were placed in mid-May of 2010 but only the second flight was seen (14 July - 29 July). Only 14 adults were captured (figure 1). In 2011, traps were located in time to detect the first flight but captures were very low over the season (figure 1).

At Fonzaso, two flights were observed in 2010, the first was already started when the traps were placed and the second started on third of July, few adults were captured (figure 2). In 2011, two flights were seen, the first one from 13 April to 2 May, and the second from 14 June to 8 July. At the flight peaks 13 (for the first flight) and 14 males (for the second flight) were captured (figure 2).

At Valdobbiadene 1, three flights were seen in 2010, the first was detected on 8 May, the second on 21 June, while the third on 23 August (figure 3). The higher number of captures (17) was recorded in late June. In 2011, three flights took place, the first started on 11 April, the second on 10 June and the third on 23 August. The highest number of captures (21) was recorded on 26 April and 20 June (figure 3).

At Valdobbiadene 2, two flights were observed in 2010 (figure 4). The first one was already started when the traps were placed (10 May) and the second started on 18 June. The highest number of captures was recorded on 27 June at the peak of the second flight. In 2011 three flights were seen, the first started on 11 April, the second on 8 June and the third on 23 August (figure 4). In 2012, three flights were recorded again. The beginning of the three flights was registered on 25 April, 20 June and 12 August respectively (figure 4).

At Pernumia, *E. ambiguella* was not detected in 2010. In 2011 few individuals were captured during the vegetative season but no clear flights were seen (figure 5).

At Roncade, few individuals were captured during April and May of 2010 and some others later. In 2011, the second and third flights started on 14 June and 8 August, respectively, but both were characterised by few captures (figure 6).

At Meolo, the traps for *E. ambiguella* were placed only in 2011 and 2012. In 2011, three flights were observed, the first one detected from 11 April, the second started on 8 June and the third on 26 July. In 2012, the only captures of second and third flights were available. The second flight started on 26 June and the third on 24 July. Captures appeared to be higher in 2011 than in 2012 (figure 7).

In 2011 all the second flights started earlier than in 2010 and 2012 by ten days (Valdobbiadene 2) to 19 days (Fonzaso). The third flight, on the other hand, started on the same days over the three years or significantly earlier in 2012 than in 2011 (see Valdobbiadene 2). Meolo and

Valdobbiadene 2 had the earliest second flights and Valdobbiadene 2 the highest captures compared to other sites.

Lobesia botrana

No *L. botrana* captures were detected in Feltre and few adults were captured at Fonzaso (figures 1 and 2).

At Valdobbiadene 1, three flights were noticed in 2010 (figure 3). The first flight was already started when traps were placed. The second flight started on 1st of July and the third on 28 August. In 2011, only two flights were seen, the first one started on 16 April and the second on 19 June (Figure 3).

At Valdobbiadene 2, three flights were registered during 2010, 2011 and 2012 (figure 4). In 2010, the first flight probably started before traps were placed. The second flight started on 18 June and the third on 25 August. In 2011, the second flight started on 17 June and the third on 23 August. In 2012, the first flight started in late April, the second on 20 June and the third on 7 August. There were few individuals in October.

At Pernumia, the first flight of 2010 was missed. The second flight started on 17 June, the third on 2 August and the fourth started in early October (figure 5). In 2011, the traps were placed earlier and the first flight occurred in April. Then captures were low. Trends suggest that the second flight started on 9 June, the third on 18 July, and the fourth on 23 September (figure 5).

At Roncade, *L. botrana* showed three flights in 2010. The first flight started on 23 April, the second on 17 June and the third on 4 August. In 2011, three flights took place, the first flight started on 7 April, the second on 14 June and the third on 4 August (figure 6). In 2012 the first flight lasted from mid April to early June. The second flight started on 24 June and the third on 5 August. However, there were additional captures from mid-September to early October (figure 6).

At Meolo, observations in 2010 started with second flight (not reported in figure 7) while three flights were confirmed in 2011 (figure 7); the first started in early April, the second on 8 June and the third on 2 August. In 2012 the first flight was missed, the second started on 26 June and the third on 22 July (figure 7).

In all the locations the second flight of *L. botrana* was earlier in 2011 than in 2010 and 2012. The only big variation was observed in Meolo between 2011 and 2012. Contrasting results emerged for the third flight that was earlier in 2011 or in 2012. Regarding the locations, the second and third flights started earlier in Pernumia, Meolo and Roncade.

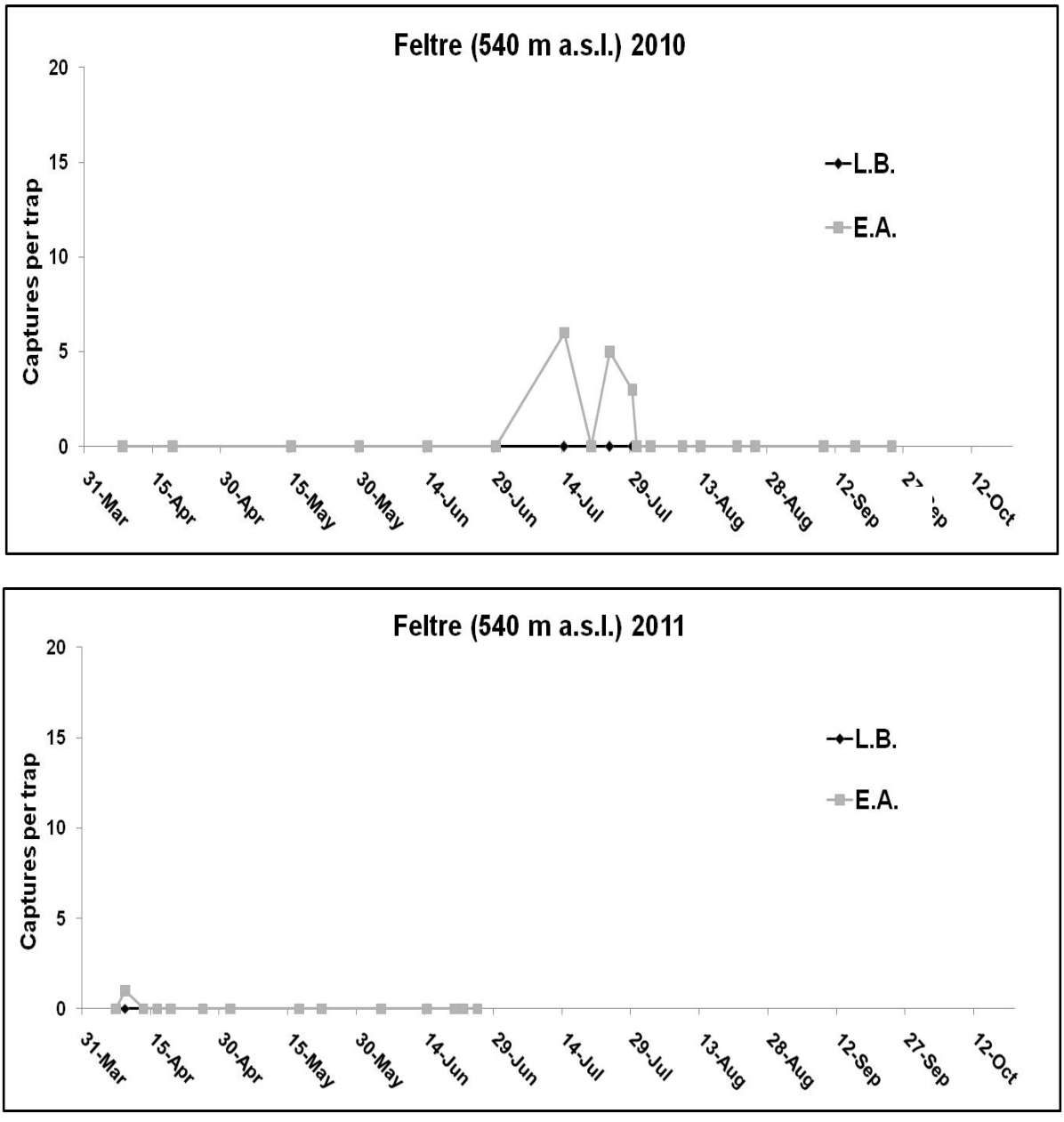


Figure 1- Phenology of *L. botrana* (L.B.) and *E. ambiguella* (E.A.) at Feltre for 2010 and 2011.

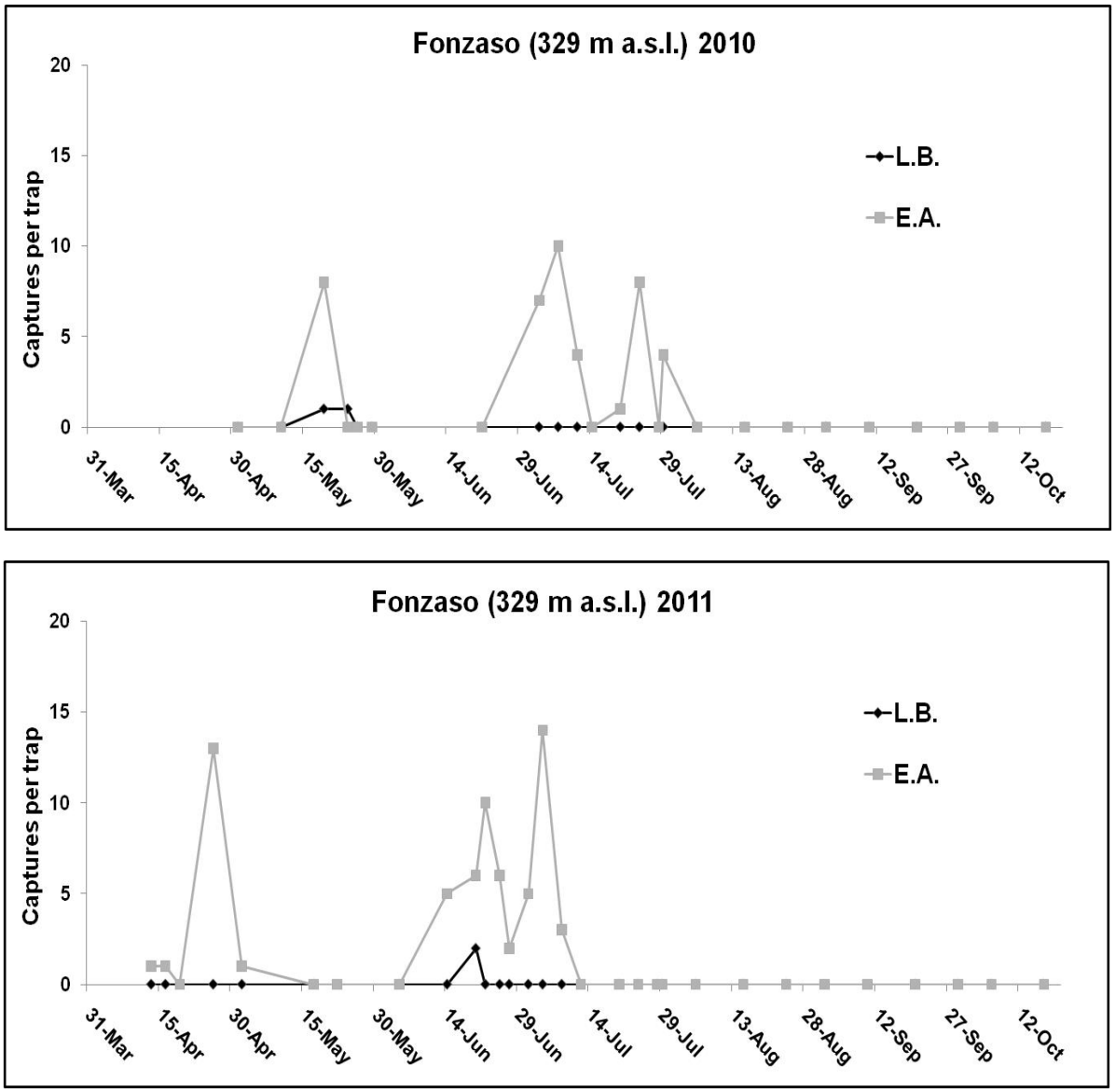


Figure 2- Phenology of *L. botrana* (L.B.) and *E. ambiguella* (E.A.) at Fonzaso for 2010 and 2011.

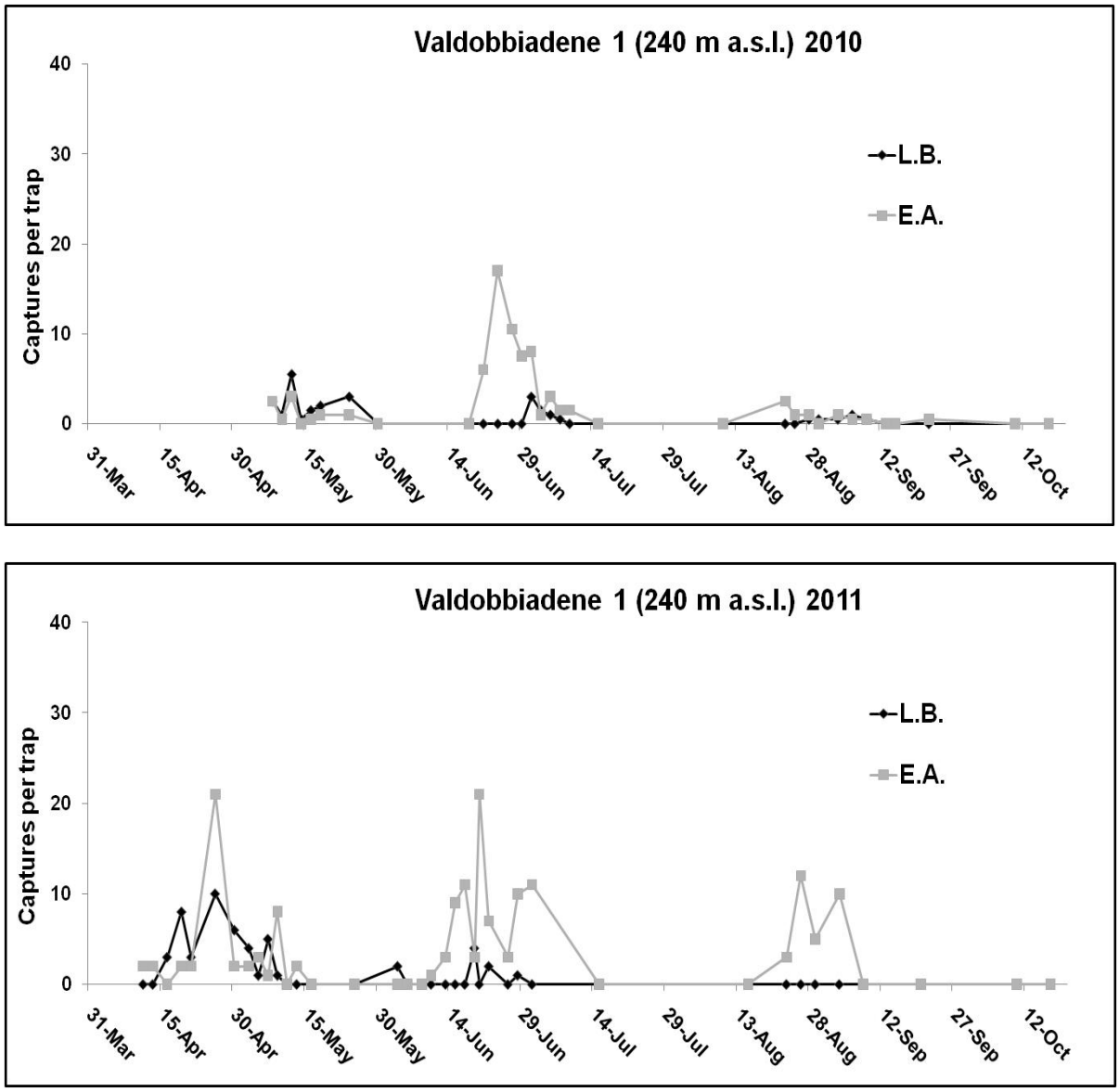


Figure 3- Phenology of *L. botrana* (L.B.) and *E. ambiguella* (E.A.) for 2010 and 2011 at Valdobbiadene1.

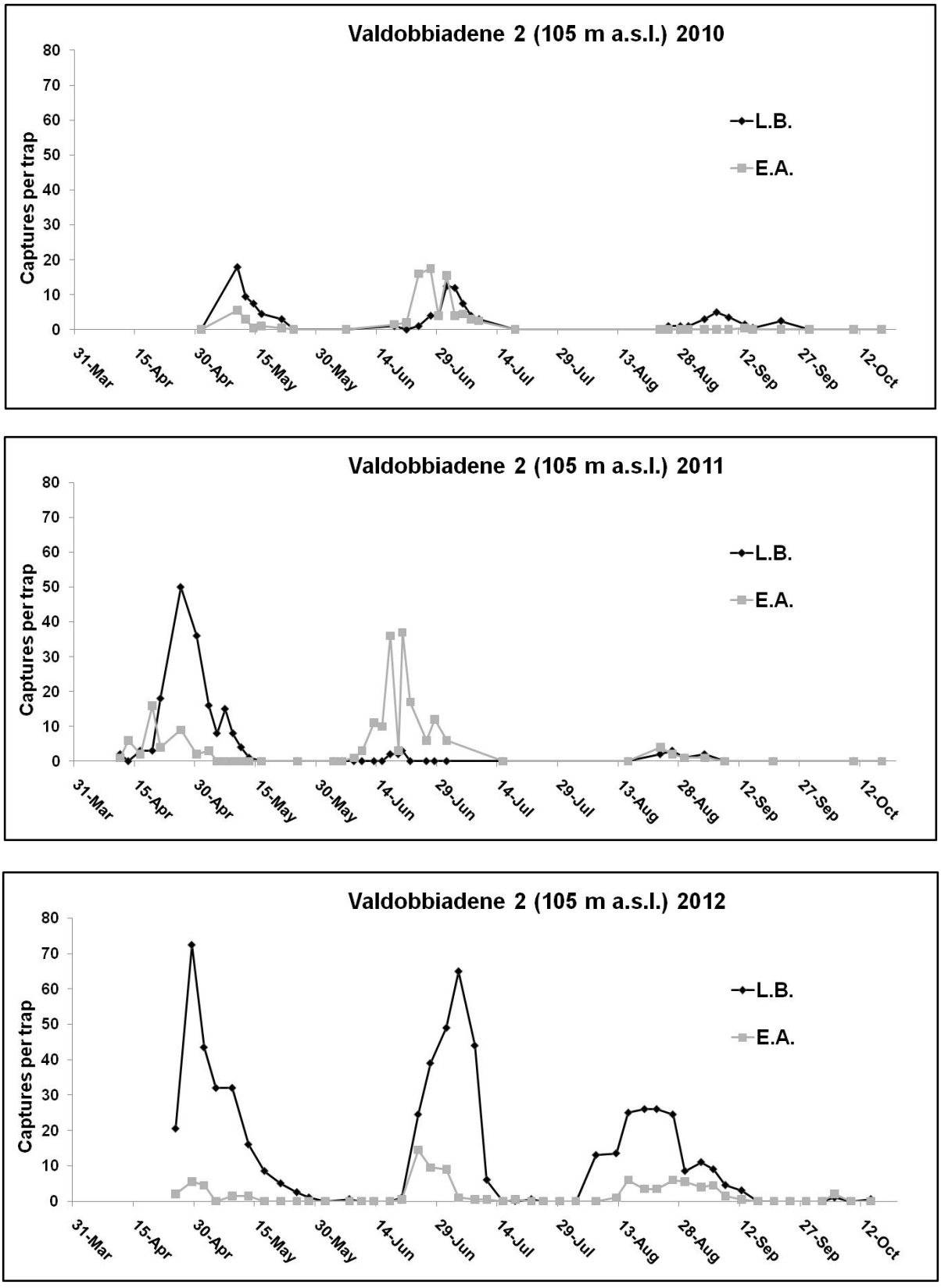


Figure 4- Phenology of *L. botrana* (L.B.) and *E. ambiguella* (E.A.) for 2010-2012 at Valdobbiadene2.

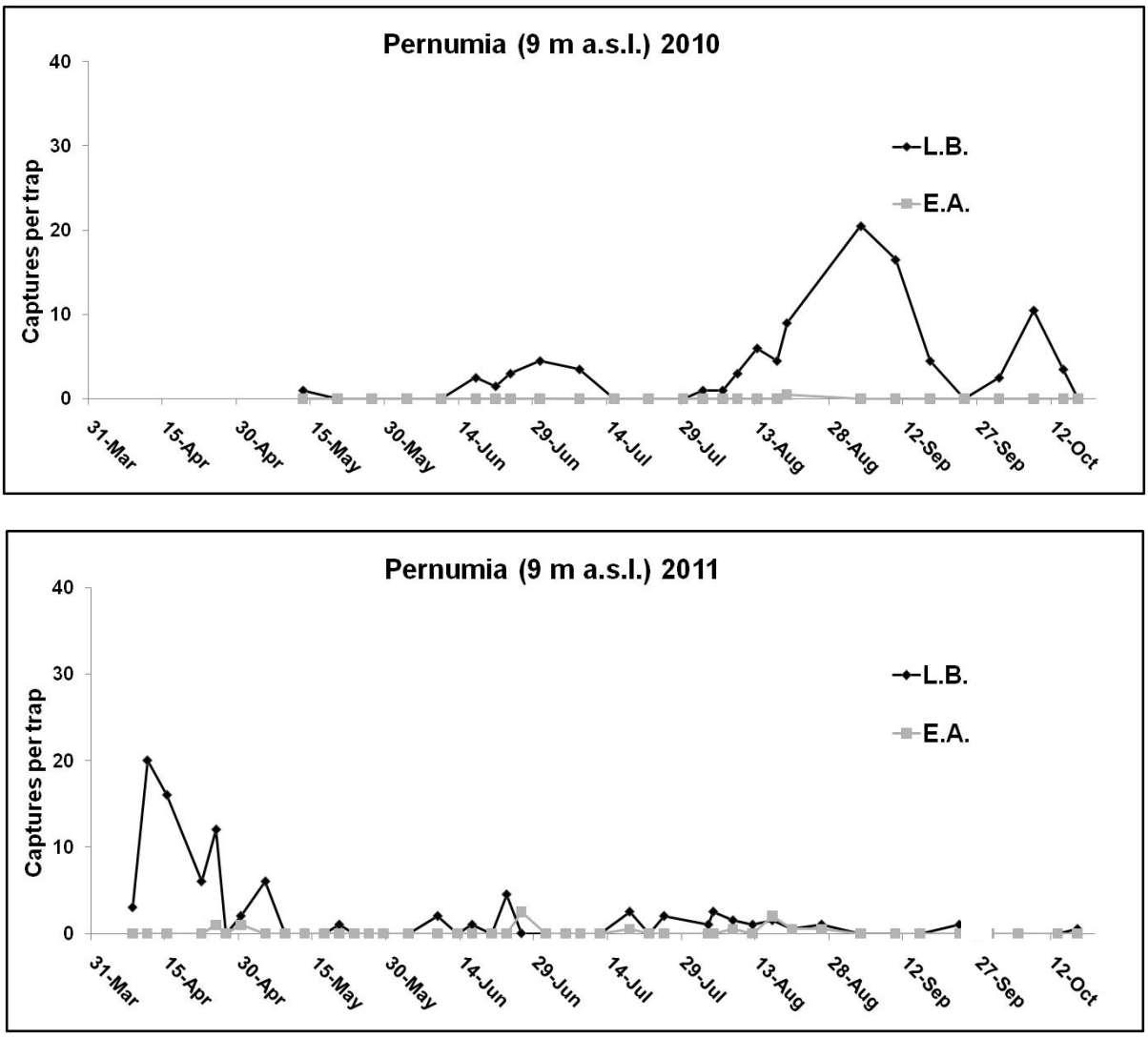


Figure 5- Phenology of *L. botrana* (L.B.) and *E. ambiguella* (E.A.) at Pernumia for 2010 and 2011.

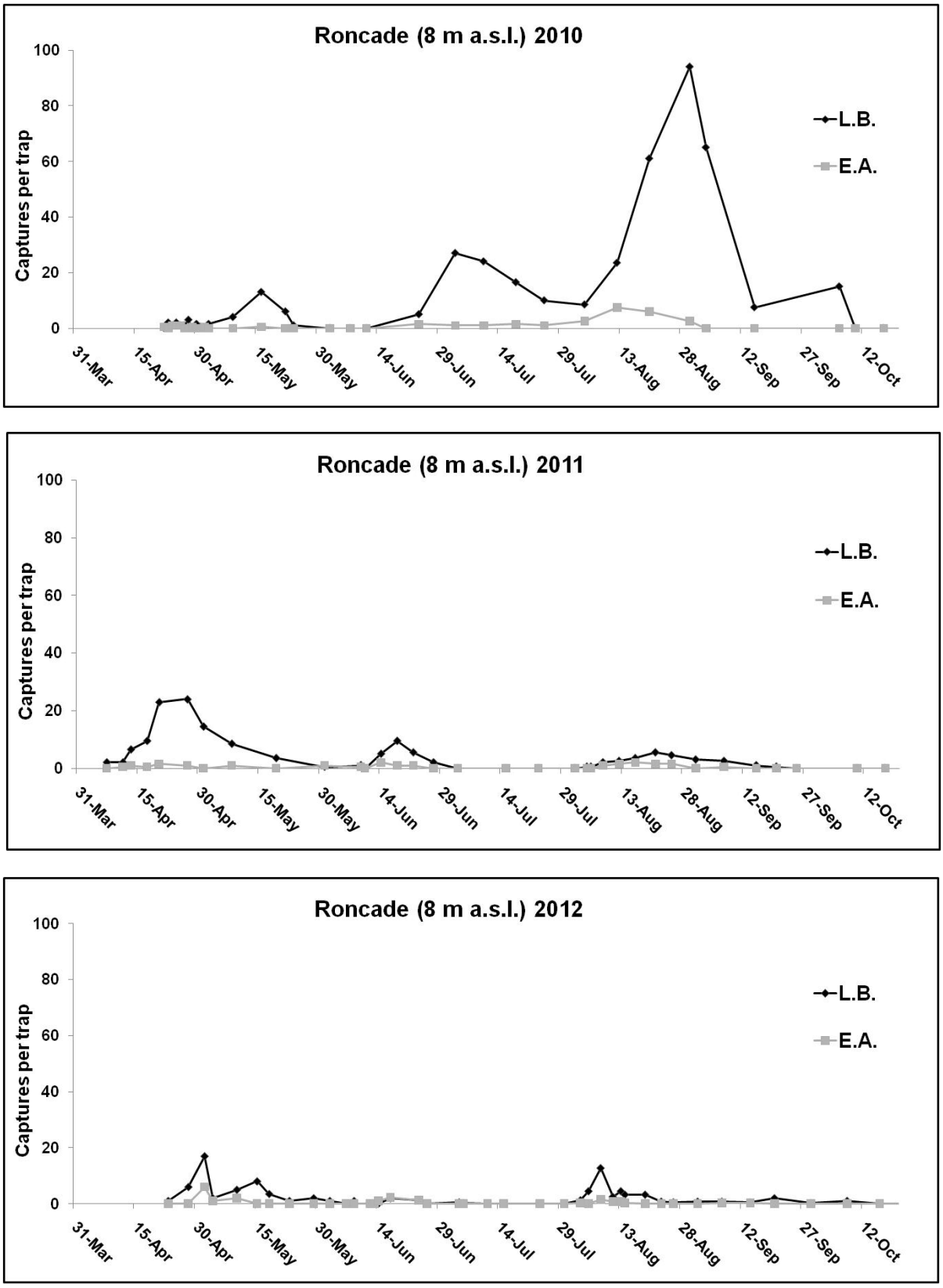


Figure 6- Phenology of *L. botrana* (L.B.) and *E. ambiguella* (E.A.) at Roncade for 2010-2012.

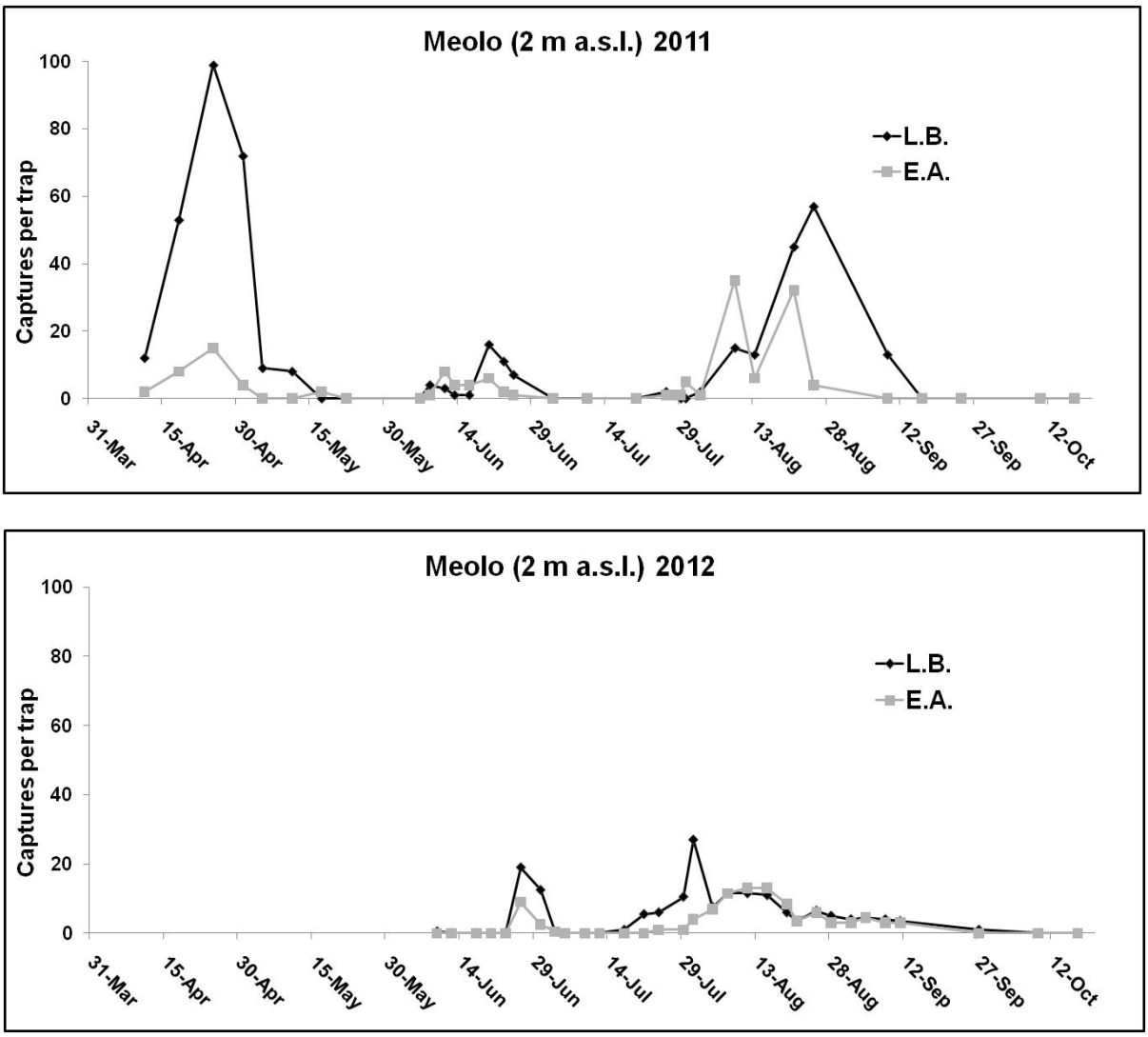
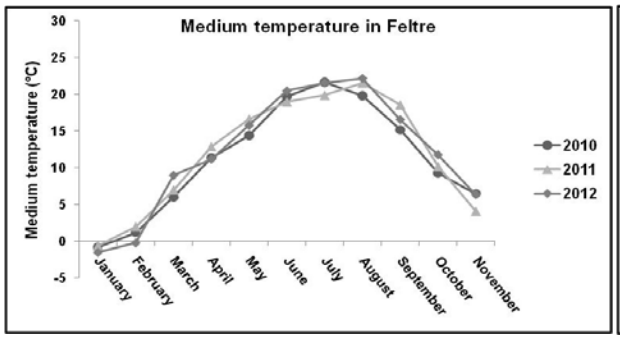


Figure 7- Phenology of *L. botrana* (L.B.) and *E. ambiguella* (E.A.) in Meolo for 2011 and 2012.

Trends in temperature

The highest temperatures were recorded in plain sites (Pernumia, Roncade and Meolo). In most sites temperatures of March were higher in 2012 than in other years. On the other hand temperatures of April and May were often higher in 2011. Temperatures recorded in June and August were higher in 2012. In July relatively high temperatures were detected in 2010 and 2012 than in 2011 (figure 8).


Medium temperature in Feltre
Medium temperature (°C)
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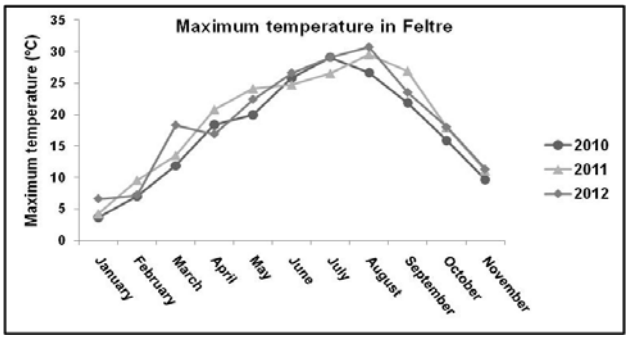
Maximum temperature in Feltre
Maximum temperature (°C)
35
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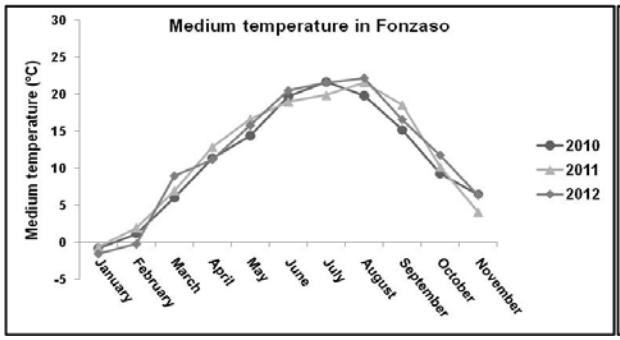
Medium temperature in Fonzaso
Medium temperature (°C)
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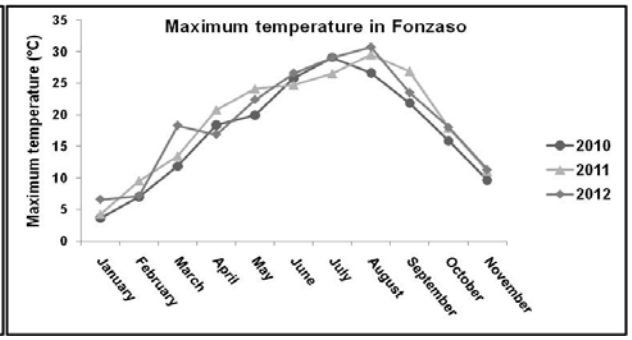
Maximum temperature in Fonzaso
Maximum temperature (°C)
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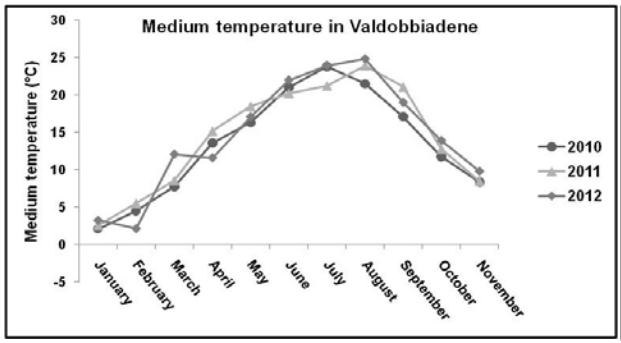
Medium temperature in Valdobbiadene
Medium temperature (°C)
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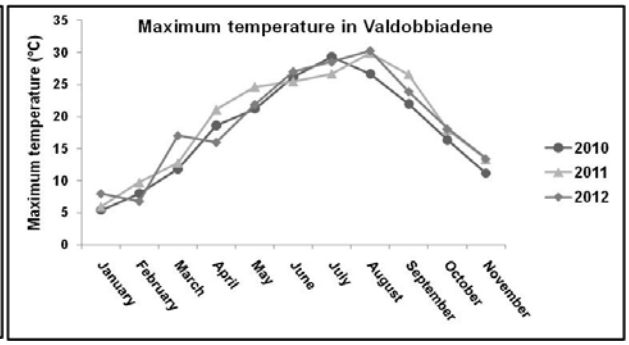
Maximum temperature in Valdobbiadene
Maximum temperature (°C)
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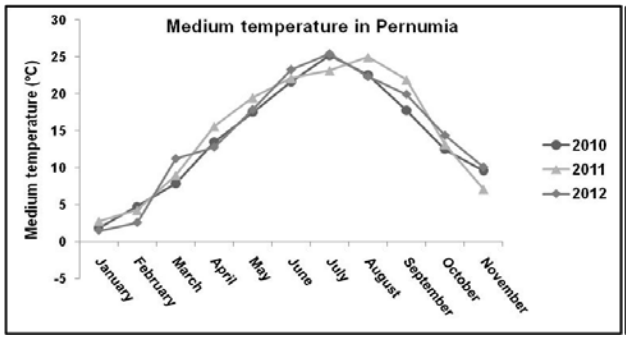
Medium temperature in Pernumia
Medium temperature (°C)
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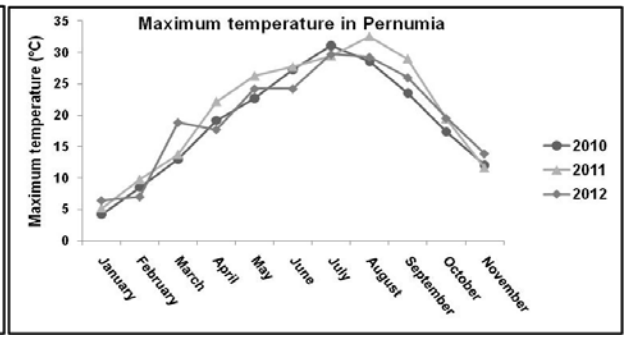
Maximum temperature in Pernumia
Maximum temperature (°C)
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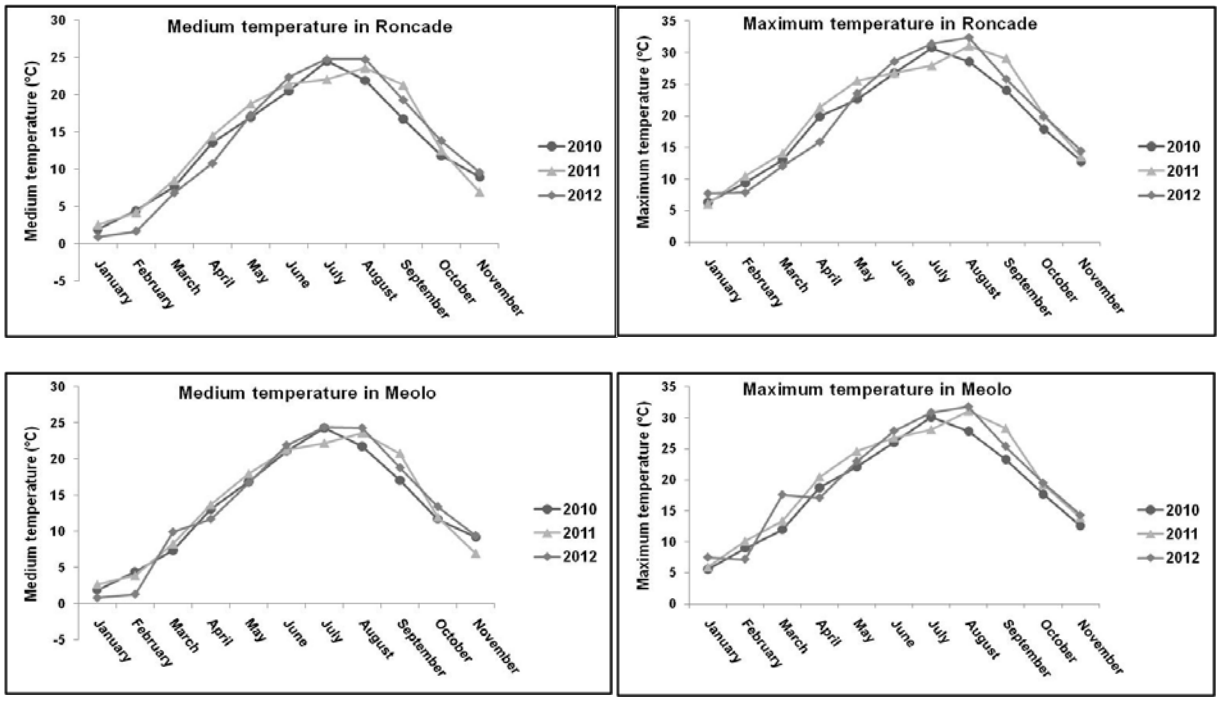

Figure 8- Medium and maximum temperatures in the experimental sites during 2010-2012

Relations between grape berry moth phenology and temperatures

Only Valdobbiadene sites were considered in relationships involving *E. ambiguella*. A significant relation was found between the beginning of the second flight and medium or maximum temperatures. This relation was also significant for the cumulated 50% of captures and medium and maximum temperatures (figure 9).

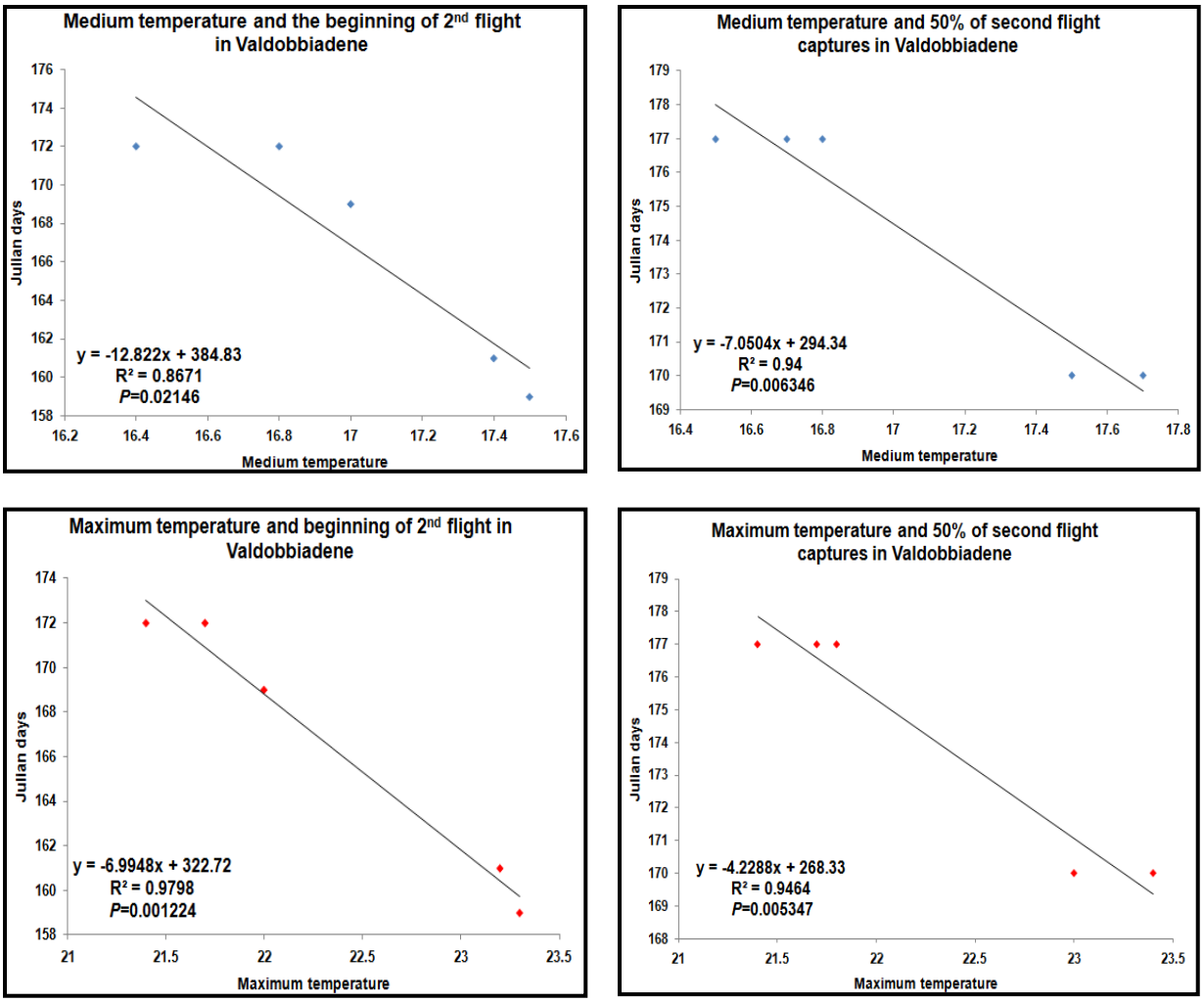

Figure 9- Relation between 2nd flight of *E. ambiguella* (beginning and 50% capture) and medium and maximum temperature (°C)

The relations between the second flight of *L. botrana* (beginning and 50% of second flight captures) and the medium and maximum temperatures were studied considering Meolo, Pernumia and Roncade. The relation between the beginning of second flight and temperature was significant as shown in figure 10.

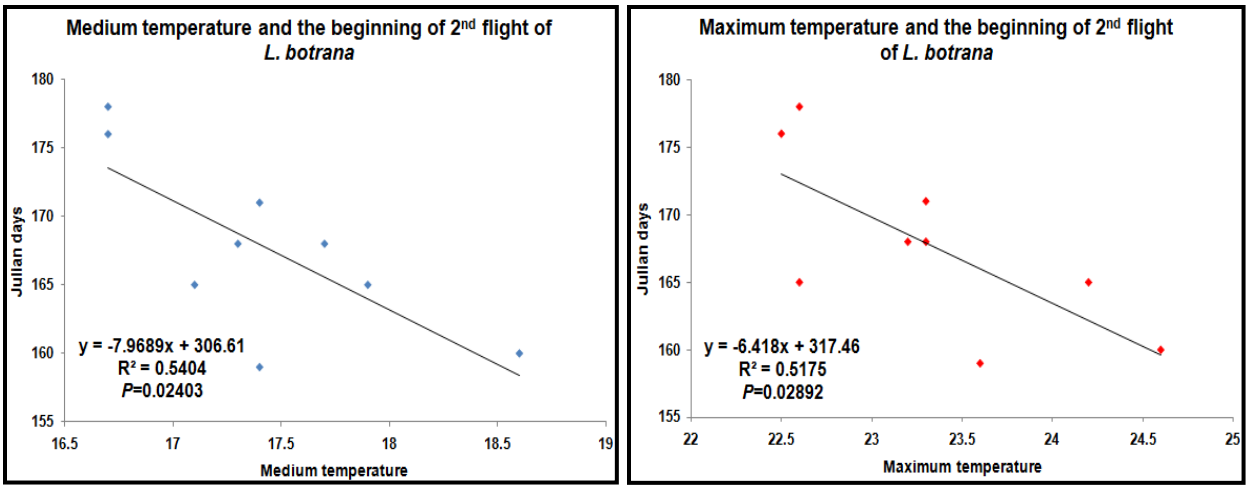

Figure 10- Relation between the beginning of the 2nd flight of *L. botrana* and medium and maximum temperature (°C).

Nevertheless, the relation between medium and maximum temperatures and the date of 50% of the second flight captures was not significant.

Discussion

The results of this study showed that *E. ambiguella* was dominant in the hilly areas (Feltre and Fonzaso), while *L. botrana* in the plain areas (Pernumia, Roncade and Meolo). In two close hilly sites (Valdobbiadene 1 and 2) both moths co-existed at significant levels and their fluctuations followed different trends. At Valdobbiadene 1, *E. ambiguella* was dominant over *L. botrana* but the latter became dominant at Valdobbiadene 2 in 2012. Probably temperature contributed to this trend as relatively high temperatures favour more *L. botrana* than *E. ambiguella* (e.g., Stellwaag 1928; Milonas *et al*., 2001; Gallardo *et al*., 2009; Martin-Vertedor *et al*., 2010; Caffarra *et al*., 2011; Gutierrez *et al*., 2012). However, temperature is not the only factor to explain the presence of the two species and their fluctuations through the years and the different sites. Other factors could be also involved such as the interaction with the host plant, pesticide pressure, cropping system, landscape features, etc. (e.g., Pavan *et al*., 2006; Moreau *et al*., 2008; Sciaretta *et al*., 2008).

The phenology of grape berry moths in the three-year study showed some variation probably affected by climatic conditions and altitude. *E. ambiguella* was able to develop two generations

at Fonzaso and three generations in most of the sites. However, few males were recorded at Feltre where viticulture is poorly developed because of cold winters. For *L. botrana*, three generations were accomplished in most of the sites, but four adult peaks were observed at Pernumia. The occurrence of four flights for *L. botrana* is not necessarily associated with four generations. The duration of the third flight of *L. botrana* was sometimes unusually long (see at Roncade) suggesting a larval aestivation phenomenon, these larvae remain inactive while the remaining continues developing (Marchesini E., unpubl. data).

The most interesting data concerned the second and third flights. Regarding *E. ambiguella* the earliest second flights were detected at Meolo and Valdobbiadene 2 but male numbers reached significant levels only in the latter site. Regarding *L. botrana* the second and third flights started earlier in Pernumia, Meolo and Roncade. The relationship between berry moth phenology and temperature was found to be significant for *E. ambiguella* and the beginning of *L. botrana* second flight. These relations can be useful to point out models based on day-degrees (e.g., Touzeau, 1981; Milonas *et al*., 2001; Gallardo *et al.,* 2009). More recently, Amo-Salas *et al*. (2011) criticized the use of linear models and suggested to take into account the effect of high temperatures on *L. botrana* development to increase prediction of moth population flights.

In our study the second flights of *E. ambiguella* and *L. botrana* were found to be earlier in 2011 compared with the other two years. Again, temperature could play a role to explain this trend. Temperatures of April and May were higher in 2011 than in 2010 and 2012 and probably they accelerated the development of the first generation. Summer temperatures (especially in June and August) appeared to be higher in 2012 than in 2011 and 2010. As a consequence the third flight of both species was earlier in 2012 than in 2011 in four cases out of five.

The importance of the phenological data presented in this work lies on their use as component in building up forecasting models which could enhance the predictive ability for the occurrence of grape berry moths. Thus, achieving an enhanced control for these species by determining precisely the best timing of control. The fact that helps grapevine growers to protect their production with less pesticides application, according to current European rules.

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Chapter 3

Genetic analyses of different population of Grape Berry Moth *Lobesia botrana* (Lepidoptera: Tortricidae) with the use of microsatellite loci

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Haya AbouAssaf collected part of the data, contributed to their analysis and drafted the manuscript. This work was made in cooperation with Prof. Annette Reineke, Dr. Alberto Pozzebon, Dr. Nicola Mori and Prof. Carlo Duso.

Abstract

Lobesia botrana is a key pest for viticulture. It feeds on flowers and berries causing significant economic losses. In spite of the importance of grape berry moth, little is known about the population genetic structure of this species. In this study, six microsatellite loci were used to analyse 16 population of *Lobesia botrana* from the Middle East and Europe, to investigate their variation and structure. The results showed moderate levels of genetic differentiation among the different populations. An excess heterozygosity was noticed in most of the populations due to the mating among the populations. The populations coming from Middle East and Spain belong to different genetic clusters, while the German and Italian ones were mixed. No clear geographical effect was detected on the different studied populations.

Introduction

The grape berry moth *Lobesia botrana* (Denis & Schiffermüller) is a major pest for viticulture worldwide. It is a polyphagous insect which presence has been reported on several plant species (Bovey, 1966; Stoeva, 1982; Maher, 2002; Thiéry, 2005; Thiéry and Moreau, 2005). Besides grapevine (*Vitis vinifera* L.) it is common on *Olea europaea* L. (Savoupoulo-Soultani *et al*., 1998; Roditakis, 1988) and *Daphne gnidium* L. (Maher and Thiéry, 2006), which is thought to be the ancestral host of *L. botrana* before it shifted to grape (Balachowsky and Mesnil, 1935; Bovey, 1966; Stoeva, 1982; Thiéry and Moreau, 2005). Nowadays, this moth is widely spread in the Palearctic areas especially in the Mediterranean basin which is referred by some theories as the origin of *L. botrana*. But the origin is still under debate (Maher and Thiéry, 2006).

Lobesia botrana has two to four generations per year, depending on the temperature, photoperiod and the latitude (Roehrich and Boller, 1991; Martìn-Vertedor *et al.*, 2010). The economic importance of this moth is based on the damage caused to grape production by larval feeding on berries (Bovey, 1966; Pavan *et al*., 1998). Moreover, the larval feeding facilitates fungal infections by *Botrytis cinerea* and other species (Deseö *et al*., 1981; Badenhausser *et al*., 1999; Zahavi *et al*., 2003; Cozzi *et al.*, 2006).

Consequently, the different pest control strategies considered *L. botrana* as the main target (Moreau *et al.*, 2010). Various pesticides were used extensively for the control of this pest (Hosseinzadeh *et al*., 2011) but the raising awareness on the negative impact of chemicals and the fear of resistance development motivated the need to develop more environment friendly control methods (Saeidi and

Kavoosi, 2011). There are several examples for these alternatives, as mating disruption which has been widely used against *L. botrana* since 1994 (Feldhege *et al*., 1993; Varner *et al*., 2001; Louis *et al*., 2002). Biological control is a promising field, but it needs further studies (Marchesini and Dalla Montà, 1994; Thiéry *et al*., 2001; Chuche *et al*., 2006; Moreau *et al*., 2010).

An improved knowledge of the biology, ecology and behaviour of this species is needed to implement control measures. In this regard, studying the pest population diversity and genetic structure is essential (Amsellem *et al*., 2003; Franck *et al*., 2007; Mozaffarian *et al*., 2008). Microsatellites have been used as a powerful tool to study genetic differentiation and gene flow in insects and other animals (Loxdale and Lushai, 1998; Simard *et al*., 2000; Bailly *et al*., 2004; Keyghobadi *et al*., 2005; Endersby *et al*., 2006; Franck *et al*., 2007; Fuentes-Contreras *et al*., 2008; Chen and Dorn, 2010) because of their co-dominance and high polymorphic levels (Plaschke *et al*., 1995).

In this study, six microsatellite loci were used to investigate the genetic structure and differentiation of different natural populations of *Lobesia botrana* from the Middle East and Europe.

Materials and methods

Sampling

Lobesia botrana adults and larvae were collected from 16 vineyards located in 5 countries, during 2007-2012. The samples included one population from Spain, two populations from Syria, three populations from Israel (in particular Golan), five populations from Italy (Veneto and Tuscany regions) and five populations from Germany. The sampling locations are detailed in table 1 and figure 1. The term “population” refers here to samples taken from the same location. Adults were gathered by the use of pheromone traps and larvae were collected by direct sampling from bunches or from laboratory breeding. The individuals were preserved in pure alcohol (>95%) and stored at (-20°C) until the DNA extraction.

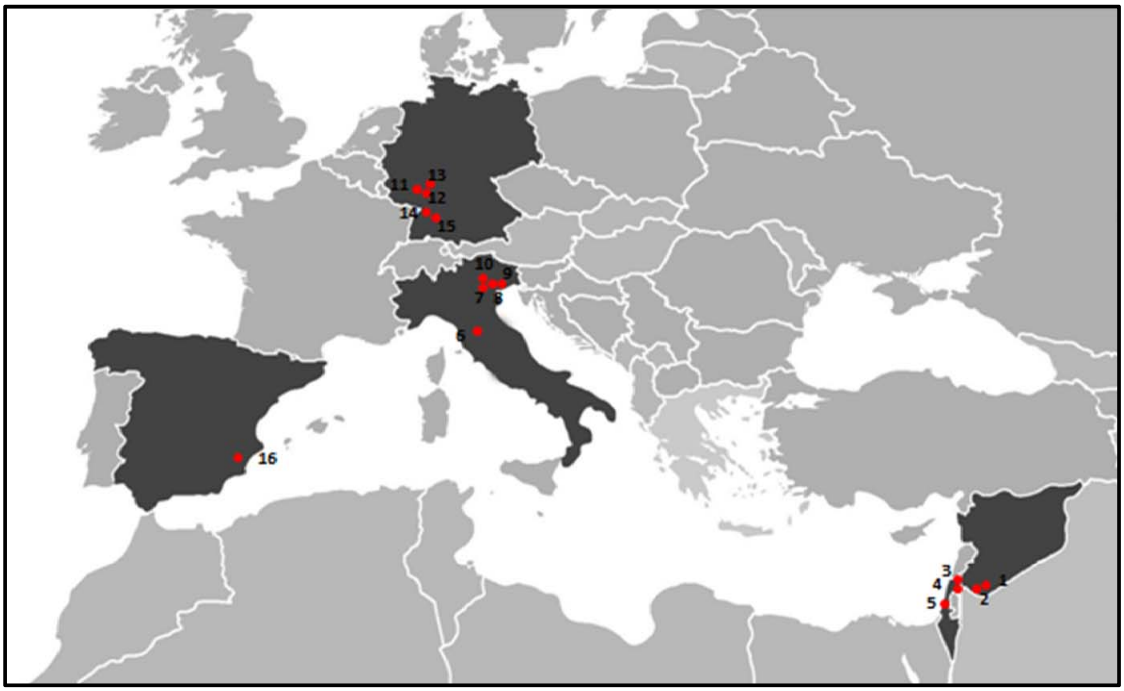

Figure 1- The sampling locations of *Lobesia botrana*.

Table 1- List of *L. botrana* samples used for the SSR analyses, their locations, date of collection and the life stage of the used insects.

Code	Country	Region	Location	Altitude m.s.l	Date	Stage	n. *
SP	Spain	Murcia	Yelca	597	2011	Adults	11
SYK	Syria	Swieda	Kafr	1352	2010	Adults	21
SYA	Syria	Swieda	Arman	1380	2010	Adults	12
PLGa	Israel	Lower Galil	Maccia	458	2011	Adults	3
PHGo	Israel	High Golan	Merom Golan	981	2011	Adults	3
PSGo	Israel	South Golan	Geshur	835	2011	Adults	7
ITS	Italy	Tuscany	Siena	322	2011	Larvae	7
ITP	Italy	Veneto	Pernumia	3	2010	Adults	14
ITD	Italy	Veneto	Roncade dalmas	8	2011	Adults	3
ITV	Italy	Veneto	Valdobbiadene	105	2011	Adults	13
ITM	Italy	Veneto	Meolo	2	2010	Larvae	20
GLS	Germany	Hesse	Lorch, Schlossberg	740	2007	Adults	10
GH	Germany	Hesse	Heppenheim, eckweg hinten	122	2007	Adults	17
GE	Germany	Hesse	Erbach	250	2007/2010	Adults	15
GF	Germany	Baden-Württemberg	Freiburg, Jesuitenschloss	274	2007	Adults	10
GK	Germany	Baden-Württemberg	Kaiserstuhl	179.5	2007	Adults	18

* The numbers here are referring to the number of individuals used in the analyses, not to the total number of individuals/sample.

DNA extraction

The total genomic DNA was extracted from adults and larvae by using a CTAB-based method (Reineke *et al*., 1998) modified by the addition of an isopropanol precipitation step. Spectrophotometer was used to measure the DNA concentration.

PCR and microsatellite analyses

The analyses were performed in the laboratories of phytomedicine Department at Geisenheim research centre in Germany. In total, 184 individuals were tested with 6 microsatellite loci (Lobbot_0569, Lobbot_0713, Lobbot_0838, Lobbot_0993, Lobbot_2967 and Lobbot_3992). Microsatellite isolation was carried out by Ecogenics (Zurich, Switzerland) by combining biotin-enrichment and high throughput 454 pyrosequencing.

Polymerase chain reaction (PCR) amplifications were carried out with a final volume of 15µl containing 40ng of the DNA template and 5pmol of the reverse and forward microsatellite primers. The reaction took place in a thermal cycler according to a touch down profile as the following: i) Denaturation step at 94°c for 4 minutes ii) Touch-Down PCR step of denaturation at 94°C for 30 seconds then annealing at 65°C for 30 sec and extension at 72°C for 30 sec, this step is repeated for 20 times with a decrease of 0.5°C for each time iii) Normal PCR step of (94°C for 15 seconds - 55°C for 30 seconds - 72°C for 30 seconds, and with 20 times of repeating)iv) Final extension at 72°C for 10 minutes.

During the preparation of the PCR mixtures, the forward primers were dye-labelled to help distinguishing among the various PCR products in the capillary electrophoresis (Franck *et al*., 2005). To allow a fluorescent labelling of the generated PCR products, a M13(-21) tail was placed at the 5'-end of each forward primer, and a fluorescently labelled CY5 universal primer M13(-21) was added to the PCR reactions according to the method described by Schuelke (2000). The fluorescent dyes were as the following: BMN5 blue with (Lobbot_0569 and Lobbot_0713), DY-751 black with (Lobbot_0838 and Lobbot_0993) and DY-681 green with (Lobbot_2967 and Lobbot_3992).

Then, PCR products were separated and analysed through capillary electrophoresis on a GenomeLab GeXP DNA Genetic Analysis System (Beckman). Reactions were loaded as a multiplex analysis.

Most of the reactions were repeated three times to check the products of the amplification process. Allele sizes were determined using GenomeLab GeXP Version 10.2 (Beckman).

Data analyses

The microsatellite data were analysed in two ways, first by analysing each population separately, second by grouping the populations according to their geographical location. Arlequin version 3.5 (Excoffier and Lischer, 2010) was used for the analyses of both ways. Genetic variation within the populations was quantified by calculating the number of alleles, their sizes, allelic richness, and the observed and expected heterozygosities H_O and H_E respectively. Also, the deviations from Hardy–Weinberg equilibrium for each population were tested. The population differentiation was studied by calculating the global estimate of F_{ST} and population pairwise measures of F_{ST}, and their significance was also determined.

In order to investigate the genetic population structure among and within groups using hierarchical analyses of molecular variance (AMOVA), *Lobesia botrana* populations were divided into 3 groups according to geographical location (table 2).

Table 2- The groups of *L. botrana* populations for the AMOVA analyses

N. group	Name of the group	Populations in the group
1	South Mediterranean	SYK, SYA, PLGa, PHGo, PSGo
2	North Mediterranean	ITS, ITP, ITV,ITM, ITD, SP
3	Germany	GLS, GH, GE, GF, GK

Null allele frequency was estimated using Genepop 4.0.10 (Raymond and Rousset, 1995) for each locus through all the populations, based on the expectation maximization (EM) algorithm by (Dempster *et al*., 1977).

Finally, the software STRUCTURE release 2.2 (Pritchard *et al*., 2007) was used to estimate the number of genetic clusters (K) for all the European and Middle East populations all together, and after grouping them according to the geographic location as shown in table 3. STRUCTURE performs the Bayesian assignment analysis of Pritchard *et al*. (2000) to assign individuals to genetically similar clusters. An admixture model and no prior information about populations were used. Twenty independent runs were done for each value of clusters (K) (K=1-18) with a burn-in period of 10,000 iterations and 10,000 post burn-in in Markov chain Monte Carlo (MCMC) iterations for each K value. The method described by Evanno *et al*. (2005) was followed to determine the most likely number of clusters (K).

Table 3- The grouped populations used for the STRUCTURE software

n. group	Name of group	Populations within the group
1	Middle East	SYK, SYA, PLGa, PHGo and PSGo
2	Italy	ITS, ITP, ITV ITM and ITD
3	Germany	GLS, GH, GE, GF and GK
4	Spain	SP

Results

Diversity of Lobesia botrana populations

The six SSR loci scored a total of 54 alleles for the 16 *L. botrana* populations and the 184 individuals. The number of alleles for all the loci ranged from 6 (in the population GK) to 2.2 (in PLGa) (table 4). The locus Lobbot_0993 showed the highest number of alleles (16), while Lobbot_0838 showed the lowest number (6) (table 6). The 6 used SSR loci were polymorphic in the 16 populations except for the Lobbot_0838 which was monomorphic in 7 populations (PLGa, ITD, ITM, GH, GF, GK and GE). In the same context, Lobbot_0993 was monomorphic only in 1 population (PLGa), and Lobbot_3992 was also monomorphic in one population (ITD). The range of observed heterozygosity was between 0.92 and 0.62, while the average for all the populations over all the loci was 0.78 (table 4). While the expected heterozyosity ranged from 0.83 in GK to 0.56 in ITV (table 4).

All the studied populations showed negative values of F_{IS} over all the 6 loci (table 4), all the values were significant except those of (PHGo, PSGO, SP and GE). As well as, when calculating populations specific F_{IS} indices per polymorphic locus, the values were found again negative except for the locus Lobbot_3992. F_{IS} value was unavailable for the Lobbot_0993 because it is monomorphic (table 6).

The maximum likelihood estimation of null alleles frequency showed the presence of null alleles, but this frequency was generally low (table 5). However, few exceptions are noticed for some populations and microsatellite loci. For example, SP population showed high null alleles frequency with (Lobbot_2967, 0993 and 3992), which were (0.6, 0.3 and 0.1) respectively. The locus Lobbot_2967 showed the higher frequency value of 0.6 with SP as was mentioned before. While the lowest frequency was shown by both Lobbot_0713 and 0993, the value is 0.01 and it was shown with (GE and PSGo respectively). However, the mean values of null alleles frequency for all the populations ranged from 0 (in PHGo, ITD and GLS) to 0.17 (in SP) (see table 4).

Table 4- Statistical analyses for 16 *Lobesia botrana* populations expressed by mean values for six microsatellite loci. The table includes the following information: code of each population; number of individuals of each population (N); number of alleles (±standard deviation); allelic range (AR); observed (H_O) and expected (H_E) heterozygosity, population specific (F_{IS}) indices, and (Na) null alleles frequency.

Code	**N**	**A (±SD)**	**AR**	**H_O**	**H_E**	**F_{IS}**	**Na**
SP	11	5.17 (1.94)	73.17	0.62	0.70	-0.04849	0.17
SYK	21	3.83 (0.75)	86.33	0.83	0.66	-0.30545	0.01
SYA	12	5.00 (1.67)	85.67	0.87	0.74	-0.40024	0.02
PLGa	3	2.17 (1.47)	71.50	0.67	0.77	-0.26316	0.08
PHGo	3	3.67 (0.52)	85.33	0.89	0.82	-0.10345	0
PSGo	7	3.33 (1.03)	83.00	0.71	0.62	-0.16505	0.01
ITS	7	3.67 (1.03)	84.33	0.88	0.68	-0.32537	0.03
ITP	14	4.50 (1.76)	86.67	0.67	0.65	-0.47546	0.02
ITD	3	2.33 (1.37)	55.75	0.92	0.82	-0.38462	0
ITV	13	4.00 (1.67)	74.67	0.62	0.56	-0.41390	0.02
ITM	20	3.50 (2.07)	76.80	0.81	0.62	-0.50271	0.01
GLS	10	4.33 (1.51)	87.33	0.83	0.64	-0.31965	0
GH	17	4.50 (2.66)	77.20	0.89	0.68	-0.39390	0.01
GE	15	5.50 (2.07)	86.33	0.66	0.68	-0.08108	0.01
GF	10	3,67 (1.63)	76.20	0.82	0.68	-0.44828	0.05
GK	18	6.00 (2.68)	79.20	0.84	0.83	-0.20956	0.05

Table 5- Locus by population of estimated null alleles frequency

Loci	SP	SYK	SYA	PLGa	PHGo	PSGo	ITS	ITP	ITD	ITV	ITM	GLS	GH	GF	GK	GE
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0.06	0	0	0.01
3	0	0	0	NA	0	0	0	0	NA	0	NA	0	NA	NA	NA	0
4	0.31	0.09	0.12	NA	0	0.01	0.19	0	0	0.12	0	0	0	0	0.09	0.14
5	0.60	0	0	0.33	0	0	0	0.03	0	0	0	0	0	0	0	0
6	0.11	0	0	0	0	0.05	0	0.08	NA	0	0.07	0	0	0.05	0.15	0.15

* NA values are due to the monomorphic locus. The loci here are expressed with numbers from 1 to 6, see table 6 for the names of these loci.

Table 6- Number of alleles, allelic size range and the population specific F_{IS} per polymorphic locus of SSR markers in 16 *L. botrana* populations. Marker Lobbot_0838 was analysed only in 10 populations, whereas markers Lobbot_0993 and Lobbot_3992 in 15 populations.

N	Locus	Number of alleles	Allele size range (bp)	F_{IS}
1	Lobbot_0569	9	56 – 64	-0.307
2	Lobbot_0713	7	58 – 64	-0.426
3	Lobbot_0838	6	127 – 135	-0.354
4	Lobbot_0993	16	19 – 100	NA
5	Lobbot_2967	8	86 – 92	-0.195
6	Lobbot_3992	8	80 – 92	0.022

Genetic structure

The result showed a significant difference from zero for the global estimate of genetic differentiation for all the 16 populations (F_{ST}=0.07951, *P*= 0.00000+0.00000). Nevertheless, this result indicates the presence of some genetic differentiation among the populations included in this study. Out of the 120 F_{ST} pairwise values among the various populations, only 58 values were found to be significantly larger than zero at the level (α=0.05), after performing Bonferroni correction for multiple comparisons (table 7). The biggest FST value was 0.27 between the Italian population from Meolo ITM (Veneto, north-eastern Italy) and the Israelian population from low Galil PLGa. In general, the Italian populations from Meolo ITM showed a high level of genetic differentiation in comparison to the other populations, where it was found to be significant in 14 of

16 pairwise F_{ST} values. Similarly, two of the German populations (GH and GF) showed a moderate to high level of genetic differentiation from almost all the other populations, they both were significant for 12 of 16 pairwise F_{ST} values.

Table 7- Pairwise F_{ST} for *L. botrana* populations. Bold numbers indicate the significant values on the level =0.05.

		SYK	SYA	PLGa	PHGo	PSGo	SP	ITS	ITP	ITD	ITV	ITM	GLS	GH	GF	GK	GE
1	SYK	0.00000															
2	SYA	0.00997	0.00000														
3	PLGa	0.01659	0.01841	0.00000													
4	PHGo	**0.06862**	0.00860	0.06071	0.00000												
5	PSGo	**0.06459**	0.02012	0.05980	0.09412	0.00000											
6	SP	**0.06459**	**0.05029**	0.03370	0.09339	0.06621	0.00000										
7	ITS	0.06459	0.01026	0.00672	0.02334	0.04288	**0.11236**	0.00000									
8	ITP	**0.06459**	0.03275	0.04766	0.05775	0.00290	0.01358	**0.07707**	0.00000								
9	ITD	0.03584	0.05953	0.14286	0.03810	0.08655	0.08022	0.02230	0.06080	0.00000							
10	ITV	**0.09835**	**0.04684**	0.11167	0.06337	**0.07378**	0.05656	**0.05989**	0.01852	0.08720	0.00000						
11	ITM	**0.16319**	**0.15696**	**0.26773**	**0.24954**	**0.04179**	**0.15087**	**0.20305**	**0.14615**	0.01283	**0.18366**	0.00000					
12	GLS	**0.09250**	**0.03712**	0.02137	**0.09392**	**0.04973**	0.04288	**0.12922**	0.05211	0.13126	0.01490	**0.06117**	0.00000				
13	GH	**0.12077**	**0.06894**	0.13298	**0.14229**	0.00292	**0.10834**	**0.05638**	**0.07707**	0.00396	**0.09000**	**0.05475**	**0.07412**	0.00000			
14	GF	**0.13124**	**0.11266**	0.08800	**0.11707**	**0.13473**	0.09278	**0.12078**	**0.04224**	0.00189	**0.07162**	**0.25994**	**0.07510**	**0.17532**	0.00000		
15	GK	**0.08715**	0.03017	0.04095	0.06639	0.02901	**0.05672**	0.01753	**0.05024**	0.01628	**0.06387**	**0.14411**	**0.06388**	**0.04509**	**0.07340**	0.00000	
16	GE	**0.11713**	0.04311	0.01097	0.08021	0.02842	**0.06146**	0.07036	0.01113	0.10022	0.04776	**0.07952**	**0.05359**	**0.02372**	**0.08422**	0.00896	0.00000

To obtain more information about the hierarchical genetic structure AMOVA test was carried out. The studied populations were divided to three groups based on their geographical locations (table 2). AMOVA results indicate the presence of high significant difference within populations and among populations within groups. On the other hand, no significant difference was found among the grouped regions (table 8).

Table 8- The results of molecular variance analyses (AMOVA) comparing among three groups of *Lobesia botrana* populations.

Variation source	d.f	Sum of squares	Variance components	% of variation	Fixation indices	*P* value
Among groups	2	15.15	0.025	1.51	0.015 F_{CT}	0.11241+-0.00955
Among populations within groups	13	49.83	0.106	6.44	0.065 F_{SC}	0.00000+-0.00000
Within populations	352	535.57	1.521	92.05	0.079 F_{ST}	0.00000+-0.00000
Total	367	600.55	1.653			

Further analyses of *Lobesia botrana* population structure were performed using the method of Bayesian clustering to detect the possible substructure in the whole dataset. The results revealed the presence of two main genotypes groups or clusters (K=2) for all the populations considered together and after grouping them based on the geographic location (see table 3). As shown in figure 2a, the first cluster is coloured in green and includes SYK, ITM and GE. The second one is in red and it contains population ITP, GK, GF and SP. The remaining populations are mixed of those two clusters. A clearer trend was found after grouping the population in four groups. The Middle East group is coloured with red, while the Spanish population is coloured with green. The Italian and German groups are a mixture of the Spanish and Middle East groups.

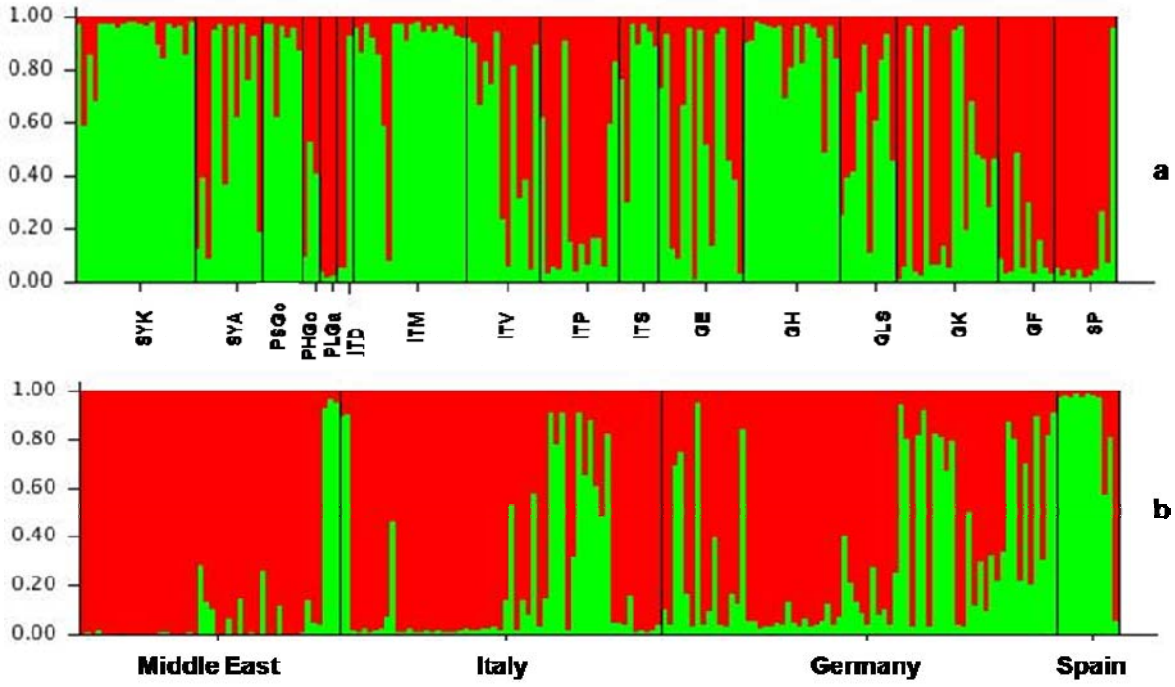

Figure 2- Populations structure as inferred in the STRUCTURE analysis with *K*=2. Each individual is represented by a vertical line with maximum of two coloured segments. a: is for all the studied populations, and b: for the grouped populations according to geographic location

Discussion

The microsatellite data revealed the presence of moderate level of genetic differentiation among the studied populations. The highest level of genetic differentiation was shown by the Italian population from Meolo (ITM), in fact this population expressed various levels of genetic differentiation even with other adjacent populations. The codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) was an object of several studies with microsatellite loci; in some of them a significant genetic

differentiation was detected (Chen and Dorn, 2010) while in others this genetic differentiation was not found (Frank *et al*., 2007; Fuentes-Contreras *et al*., 2008; Gund *et al*., 2012). At any case, the flight ability of *L. botrana* does not allow it to disperse over large distance, as was found by Roehrich and Carles (1981). Males could fly till 300 m and females are less mobile (Schmitz, 1992; Badenhausser *et al*., 1999). This low flight ability could explain the genetic differentiation among the studied populations.

Most of populations showed higher values of observed heterozygosity compared to the expected values indicating the presence of excess heterozygosity. This excess was also shown in the negative F_{IS} values. This deviation from Hardy-Weinberg equilibrium was significant in all the populations except the South and high Golan populations (PSGo and PHGo). On the other hand, the lower Galil population (PLGa) had a significant heterzygosity deficiency, while the heterozygosity deficiency in the Spanish and German Erbach (SP and GE) populations was not significant. The presence of null allele could be associated to heterozygosity deficiency (Meglécz *et al*., 2004). In this study, the Spanish and German Erbach populations showed high levels of null allele frequency with most of the loci (as shown in table 5), while the small sample size of lower Galil population (PLGa) population could lead to a true bias for the interpretation of the results. In any case, the excess of heterozygosity suggests that the populations are not well isolated among each other. This isolation breaking could have happened recently enabling the individuals to mate with other from different populations.

The AMOVA results revealed a significant differentiation among the populations and among populations within groups, due to the mating among populations. No significant variation was found among the groups which belong to different geographical areas. This result corresponds to what was found previously with excess heterozygosity, but still does not explain the significant genetic differentiation among the populations.

Two main clusters were found for all the analysed populations when considered separately and after grouping them based on the location. On the first case (when populations analysed separately), the Syrian Kafr (SYK) together with Italian Meolo and German Heppenheim (ITM and GH) populations belong to the first cluster in spite of the geographical distance among them. Whereas, the Spanish population (SP), German Kaiserstuhl (GK), German Freiburg (GF) and Italian Pernumia (ITP) belong to the other cluster. Regarding this later cluster, it only includes some European populations, yet still the distance among those populations is big. The remaining populations are mixed of those two clusters. For the second case (populations grouped), the populations from the Middle East showed a cluster differs from the one shown by the Spanish

population. Italian and German are mixture of those two clusters. This result suggests that the European populations are not strongly structured but are rather a mix of genotypes. No clear geographic separation was detected among them.

In this study, the genetic variation and structure for several populations of *Lobesia botrana* from the Middle East and Europe were described using microsatellite loci. This knowledge is valuable for the development of pest management strategies (Miller *et al*., 2003). For example, populations with highly diversified individuals could widely spread in different geographical locations, and they could feed on other plants besides grapevine. Thus, the technicians when planning for a plant protection strategy could take such information into consideration (Bournoville *et al.*, 2000). Moreover, studying the gene flow of populations could help in knowing the distance over which the population could disperse, consequently, knowing the spatial scale within which the forecasting techniques could be effective (Loxdale and Lushai, 2001). Hence, more research is needed with a bigger range of populations to understand the gene flow among them.

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Chapter 4

Observations on the phenology of *Scaphoideus titanus* (Hemiptera: Cicadellidae) in the Veneto region (North-eastern Italy)

Haya AbouAssaf collected part of the data, contributed to their analysis and drafted the manuscript. This work was made in cooperation with Dr. Alberto Pozzebon, Dr. Diego Fornasiero, Dr. Paola Tirello, Dr. Nicola Mori and Prof. Carlo Duso.

Abstract

The Nearctic leafhopper *Scaphoideus titanus* Ball is the only known vector of grapevine yellow disease Flavescence dorée. It is spread in a number of European countries and widely distributed in Italy. This study aims at studying *S. titanus* phenology in several sites in Veneto region (north-eastern Italy) in 2010-2012, in order to know the best time to apply pest control measures. First *S. titanus* nymphs appeared in May while first adults appeared from the third decade of June to mid-July, and flight until September-October. In general, *S. titanus* nymphs and adults appeared earlier in 2011 and 2012 than in 2010 with some variations among the sites depending on altitude and sun exposure. Temperature affected nymphs phenology that was partly synchronized with grapevine phenology. Among factors affecting adult phenology, temperature and altitude appeared the most important. Data reported in this work can be useful to build up a phenological model for *S. titanus*.

Introduction

The grapevine leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) is native from North America (Vidano, 1966; Maixner *et al.*, 1993). It was introduced into Europe probably during the 1950s where it appeared for the first time in France (Bonfils and Schvester, 1960). The damage of this leafhopper does not come from direct feeding on vines but through the transmission of the *Candidatus* Phytoplasma vitis 16SrV, which is the cause of the Grapevine Yellow Flavescence dorée (FD) disease (Schvester and Moutous 1961; Schvester *et al*., 1962; 1969; Carraro *et al*., 1994; Bianco *et al.,* 2001; Mori *et al*., 2002). Currently, *S. titanus* is an important grapevine pest in different European countries such as Italy, Slovenia, Switzerland, Croatia, Serbia, Hungary, Spain and Portugal (Bertin *et al.*, 2007; Schaub and Linder, 2007; Krnjaić, 2007; Seljak, 2008). In Italy, *S. titanus* is widespread except in some southern regions (Mazzoni *et al*., 2005).

Scaphoideus titanus was described for the first time in 1932 by Ball (1932). Its life cycle was firstly described in France (Schvester *et al*., 1962), followed by Vidano (1966) in Italy. Afterwards, the life cycle of this pest was described in various regions. In Veneto region (north-eastern Italy) *S. titanus* was detected by Belli *et al.* (1984) and Pavan *et al*. (1987).

Scaphoideus titanus is not widely established in all grapevine areas, and climatic conditions could explain this phenomenon (Chuche and Thiéry, 2009). Climatic conditions may affect the synchronization among the pest, the phytoplasma acquisition and the host plant development. According to Duchên and Schneider (2005), warm winters can anticipate grapevine bud burst, but it can also delay the egg hatching of *S. titanus* (Chuche and Thiéry, 2009). For *S. titanus*, similarly to species that diapause as eggs, the temperature experienced during the diapause is crucial for life cycle (Chuche and Thiéry, 2009). In other words, the low temperature during winter is useful to break the diapause and the summer temperature is needed to complete the life cycle of *S. titanus* (Boudon-Padieu and Maixner, 2007). Based on Chuche and Thiéry observations (2009), the eggs exposed to cold and moderate winters would have the same hatching pattern but beginning and peak of hatching can be anticipated after cold winters. Winter temperature could influence not only hatching time but also sex ratio, juveniles and insect population (Chuche and Thiéry, 2012). The temperature experienced by eggs determined a greater variation in hatching dynamics for females, but not for males. The dynamic of protandry of *S. titanus* is negatively associated with temperature during incubation: cold temperature induces an increase in protandry (Chuche and Thiéry, 2012). Moreover, nymphs that are exposed to warm temperatures are bigger than those exposed to cold temperatures (Chuche and Thiéry, 2012). It has been hypothesised that the thermal effects on *S. titanus* can have consequences on its spread to south Europe (Chuche and Thiéry, 2009 and 2012): 1) warm temperature may induce asynchronism between the hatching of *S. titanus* and the bud burst of grapevine determining reduced juvenile survival, 2) reduction in protandry due to warm temperatures can reduce the fitness of the species. On the other hand, climate change with global warming could have an effect on the spreading of *S. titanus*, i.e. the increasing temperature could help the insect to spread northwards (Boudon-Padieu and Maixner, 2007).

FD is a very important quarantine disease (EPPO standards, 2011) that causes a big economic damage and is considered as the most threatening among the Grapevine Yellows in Europe (Schvester *et al.*, 1969; Caudwell, 1990; Morone *et al.,* 2007). As a consequence, the control of *S. titanus* is compulsory and based mostly on pesticide use (Schvester, 1969; Pavan *et al*., 2005). However, current European Community rules require reducing insecticide use through the use of

forecasting models and alternatives to conventional pesticides (Dir. 2009/128/CE). Therefore, it is crucial to obtain detailed data on the phenology of major pests in large areas to improve pest management strategies. We studied the phenology of *S. titanus* in some areas of Veneto region in order to optimize pesticide use and obtain data useful for phenological models.

Materials and methods

Experimental sites

This study was carried out in five sites located in different districts of Veneto region during 2010-2012 (Table 1). Insecticides were not applied in the vineyards during the three experimental seasons.

Table 1- Features of sites selected for this study.

Site	District	GPS cod.	Altitude (m a.s.l.)	Cultivar
1 - Bagnoli	Padova	45° 11’ 11.77” N; 11° 53’ 11.76” E	0	Raboso Piave
2 - Portogruaro	Venice	45°48'52.27"N ; 12°43'24.94"E	6	Merlot
3 - Breganze	Vicenza	45° 42’ 43.98” N; 11° 34’ 54.49” E	112	Merlot Cabernet S.
4 - Roncà	Verona	45° 29.291’ N; 11° 17.719’E	227	Durella
5 - Mugnai	Belluno	46° 0'57.64"N ; 11°51'43.14"E	308	Pavana Bianchetta

Insect sampling

Nymphs were sampled by checking the lower surface of leaves close to the cordon/trunk and then collected using a vacuum. A total of 100 leaves were checked weekly to assess nymph stage and density. Sampling took place from May to August in each year. Nymph stages were identified using a stereoscope on the base of morphological characteristics reported in Della Giustina *et al*. (1992). Adults were sampled by using yellow sticky traps (Serbios Super Color®). Traps were checked and renewed weekly from June to October.

Climatic data

The minimum, maximum and medium temperatures were provided by ARPAV (Agenzia Regionale per Prevenzione e Protezione Ambientale del Veneto) from the nearest weather station to each site. The greatest distance between investigated vineyards and the correspondent weather stations was 3.6 km. Averages of medium and maximum temperatures from the 1st of January till the date of the first nymph and adult captures were calculated. Similarly, averages of medium and maximum temperatures from the 1st of January till the date when cumulated 50% nymph and adult populations were calculated. Regression analyses were done using R software release 2.15.0 (R Development Core Team, 2011), to study the relation between temperature and the date of first nymph and adult appearance, and the cumulated 50% nymph and adult populations. Those dates were expressed by Julian days. Degree days based on temperatures higher than a threshold of 8.7°C were also calculated.

Results

The phenology of Scaphoideus titanus

In 2010, the first *S. titanus* nymphs appeared in site 1 (19 May), while the first adults were recorded in site 4 (2 July). In site 3, *S. titanus* nymphs and adults appeared later compared to other sites (26 May for nymphs and 14 July for adults). The 50% of cumulated nymph and adult populations was reached earlier in site 2 (6 June and 3 July respectively (table 2)). The last 50% cumulated nymph population was registered in site 4 (23 June), while the last 50% of cumulated adult population (15 August) was observed in the site 5.

In 2011, the first nymphs appeared on 6 May (site 1 and 4) and the first adults on 24 June (site 4). On the other hand, nymphs and adults appeared later (26 May and 8 July, respectively) in site 5. The 50% of cumulated population was reached earlier for nymphs and adults in site 3 (4 June and 20 July respectively) for adults. The last 50% cumulated nymph number was seen in site 2 (21 June) and site 5 for the last in adult cumulating number (10 August) (table 2).

In 2012, nymphs appeared earlier (18 May) in sites 1, 3 and 4 than the remaining sites. For adults, site 2 registered the earliest appearance (5 July). Nymphs appeared later in site 5 (26 May), and adults in site 3 (16 July). The 50% of cumulated population was reached earlier in site

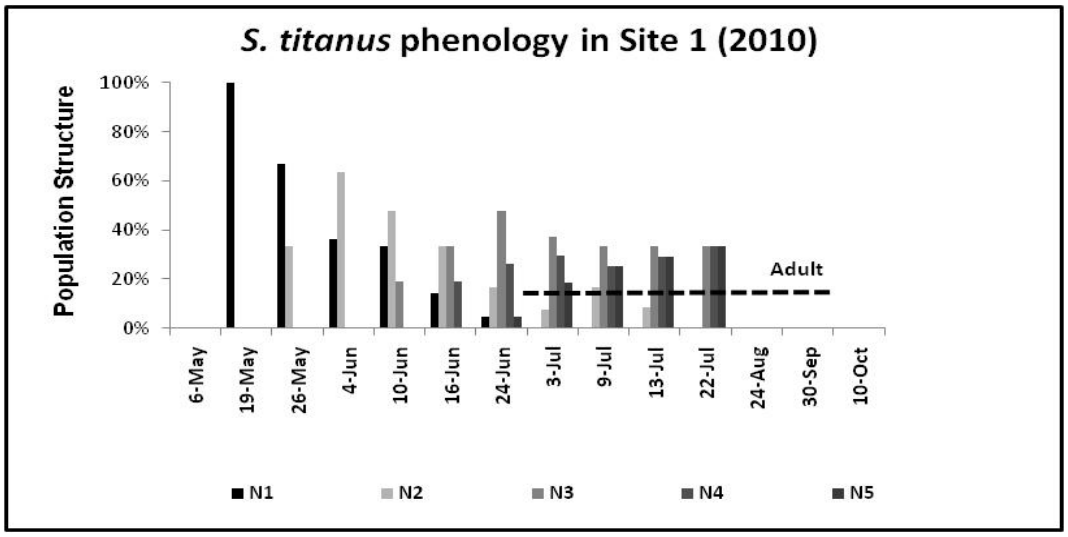
2 for nymphs (14 June) and in site 5 (16 July) for adults. Site 3 was the last in cumulating nymph numbers (28 June) and site 4 was the last in cumulating adult numbers (7 August) (table 2).

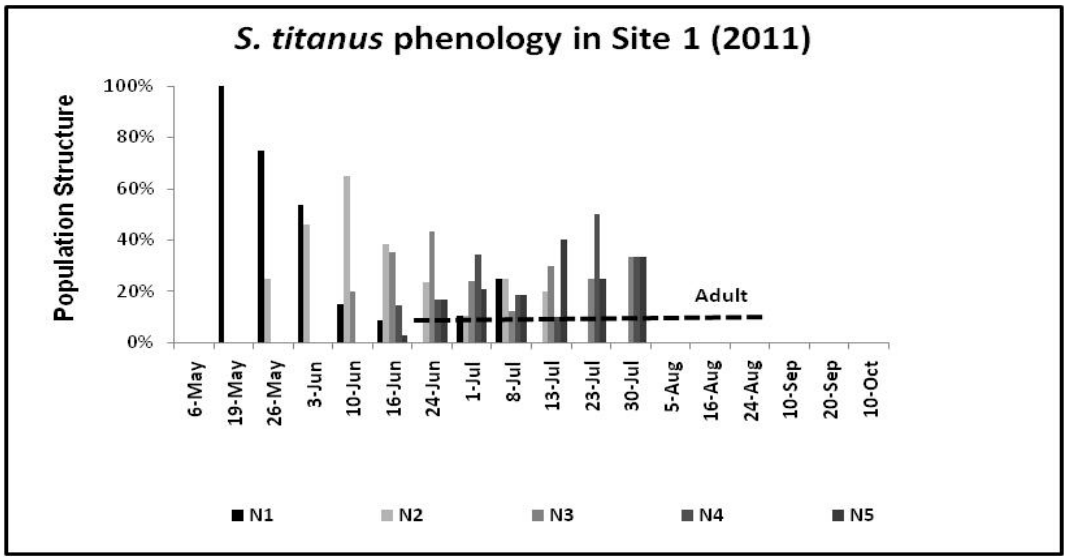
No great variation was found in the appearance dates of *S. titanus* nymphs and adults in most of the locations during 2010-2012. In general, nymph and adult appearance was earlier in 2011 and 2012 than in 2010 (figure 1). In nymphs' case, the only significant difference in appearing date among the three years was noticed in site 4, where nymphs appeared in about 12-15 days earlier in 2011 than in 2010 and 2012. Adults appeared earlier in 2011 than in 2010 and 2012 in four out of five sites; the only exception was registered in site 5, where adults appeared on the same day in both 2010 and 2011. Sites 1, 3 and 4 were associated with marked differences in adult appearance dates. In those sites the adults appeared in 2011 earlier by 15-20 days than in 2012.

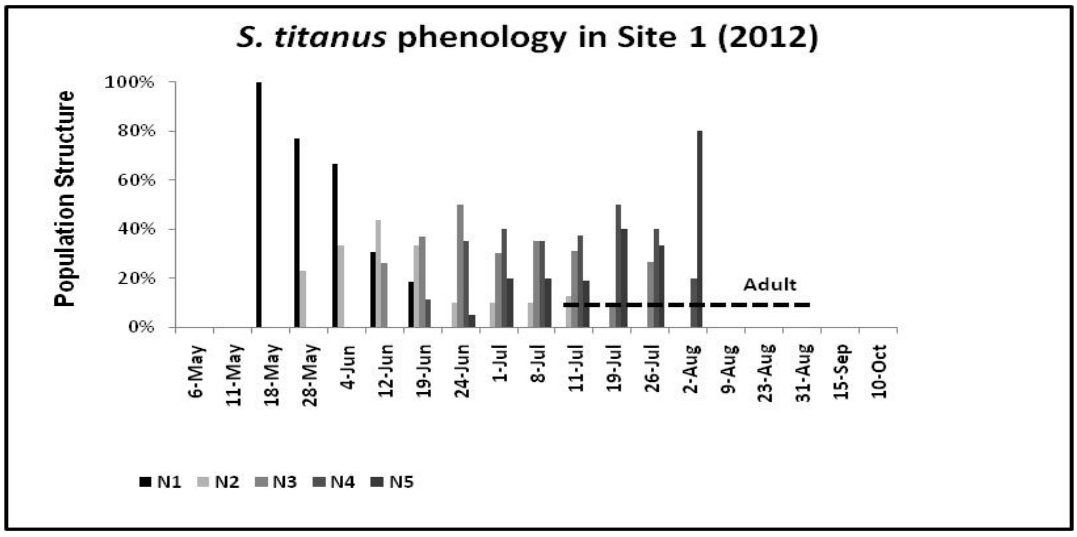
Big variation was registered in the 50% of cumulated nymph population in most of the locations during the investigated period: in site 2 it was obtained 7-15 days earlier in 2010 than in 2012 and 2011 respectively, in site 3 it was registered 15-23 days before in 2011 than in 2010 and 2012 and in site 1 it was reached 12-13days before in 2012 than 2010 and 2011. Site 5, showed small variation of 5 days between 2010 and 2011, while this variation reached 25 days to a month between 2012 and 2011 and 2010 respectively (table 2).

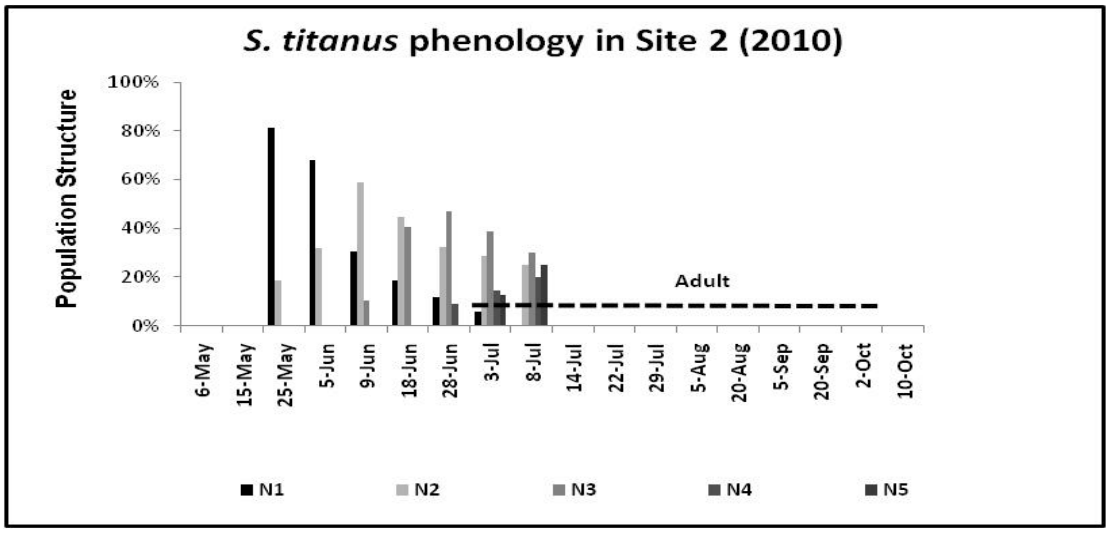
Table 2- Data for the 50% of cumulated nymphs and adults populations.

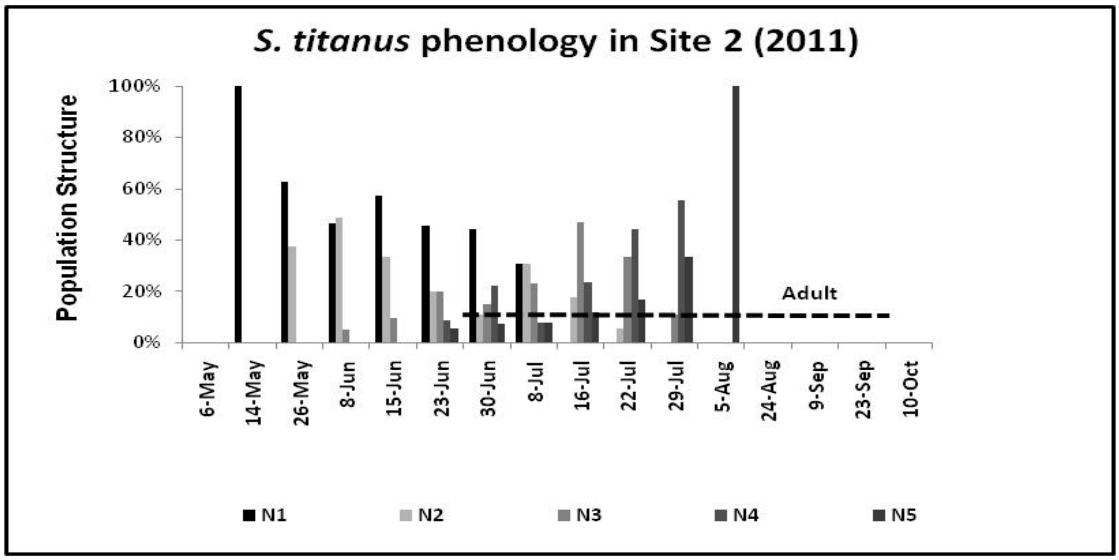
Site	Stage	2010	2011	2012
Bagnoli	**Nymph**	15-Jun	14-Jun	14-Jun
	Adult	20-Jul	26-Jul	31-Jul
Portogruaro	**Nymph**	06-Jun	21-Jun	13-Jun
	Adult	3-Jul	02-Aug	18-Jul
Breganze	**Nymph**	19-Jun	04-Jun	27-Jun
	Adult	22-Jul	20-Jul	31-Jul
Ronca	**Nymph**	23-Jun	20-Jun	22-Jun
	Adult	29-Jul	27-Jul	07-Aug
Mugnai	**Nymph**	11-Jun	12-Jun	24-Jun
	Adult	15-Aug	10-Aug	15-Jul

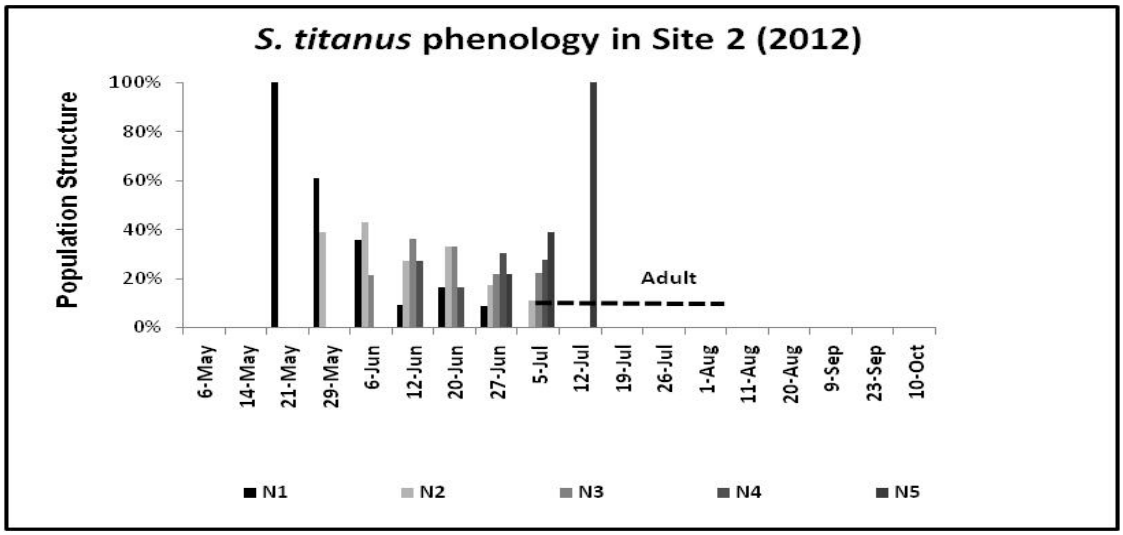

S. titanus phenology in Site 1 (2010)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
19-May
26-May
4-Jun
10-Jun
16-Jun
24-Jun
3-Jul
9-Jul
13-Jul
22-Jul
24-Aug
30-Sep
10-Oct
N1
N2
N3
N4
N5

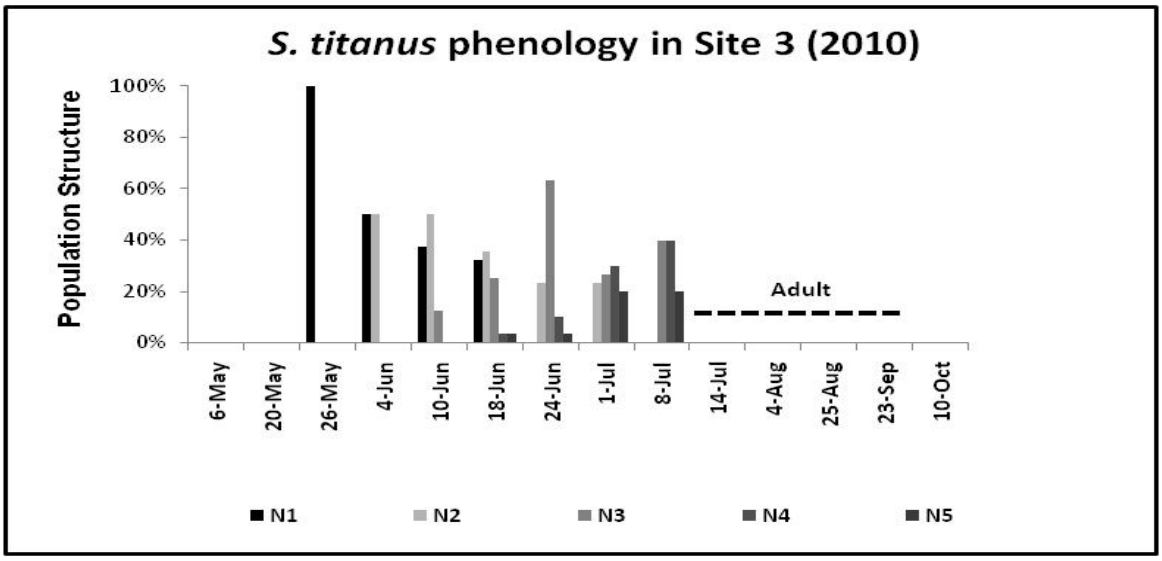

S. titanus phenology in Site 1 (2011)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
19-May
26-May
3-Jun
10-Jun
16-Jun
24-Jun
1-Jul
8-Jul
13-Jul
23-Jul
30-Jul
5-Aug
16-Aug
24-Aug
10-Sep
20-Sep
10-Oct
N1
N2
N3
N4
N5

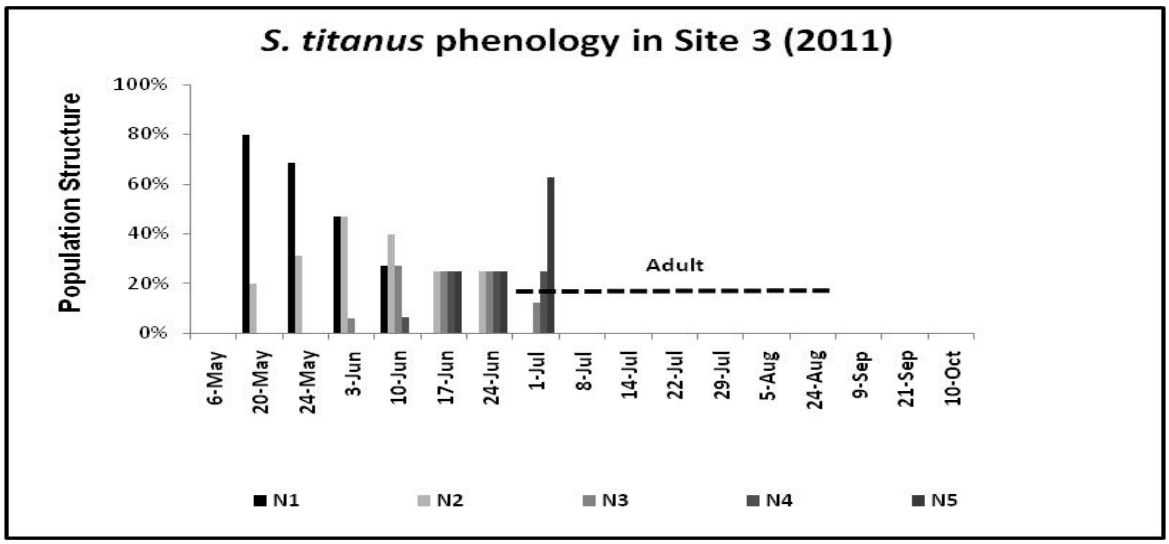

S. titanus phenology in Site 1 (2012)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
11-May
18-May
28-May
4-Jun
12-Jun
19-Jun
24-Jun
1-Jul
8-Jul
11-Jul
19-Jul
26-Jul
2-Aug
9-Aug
23-Aug
31-Aug
15-Sep
10-Oct
N1
N2
N3
N4
N5

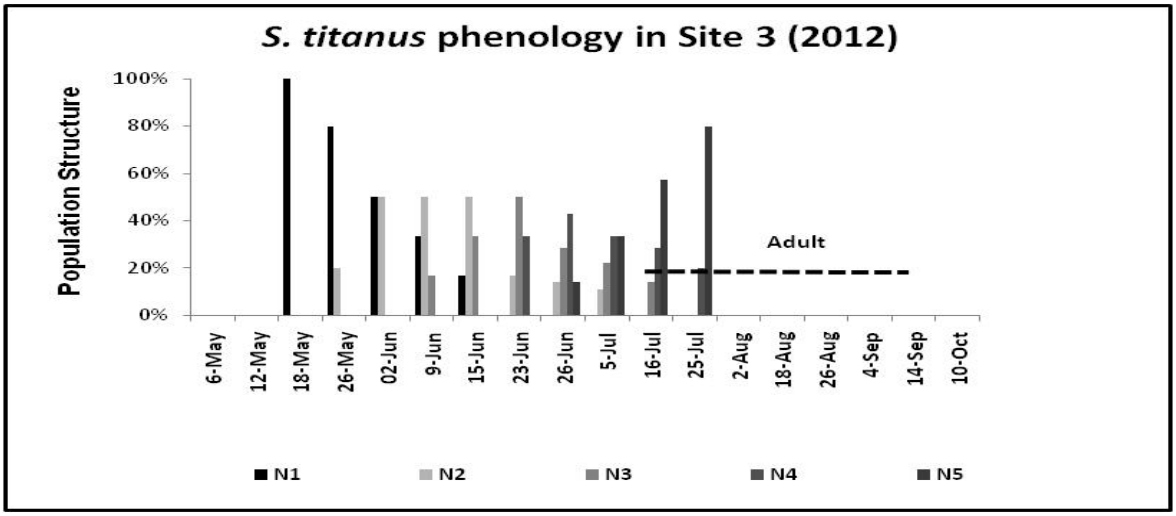

S. titanus phenology in Site 2 (2010)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
15-May
25-May
5-Jun
9-Jun
18-Jun
28-Jun
3-Jul
8-Jul
14-Jul
22-Jul
29-Jul
5-Aug
20-Aug
5-Sep
20-Sep
2-Oct
10-Oct
N1
N2
N3
N4
N5

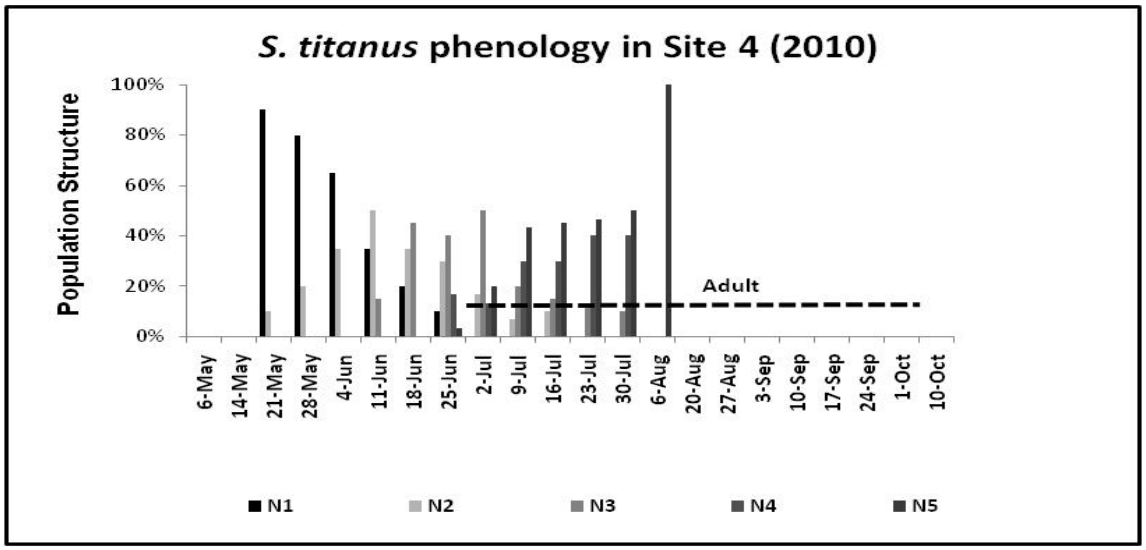

S. titanus phenology in Site 2 (2011)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
14-May
26-May
8-Jun
15-Jun
23-Jun
30-Jun
8-Jul
16-Jul
22-Jul
29-Jul
5-Aug
24-Aug
9-Sep
23-Sep
10-Oct
N1
N2
N3
N4
N5

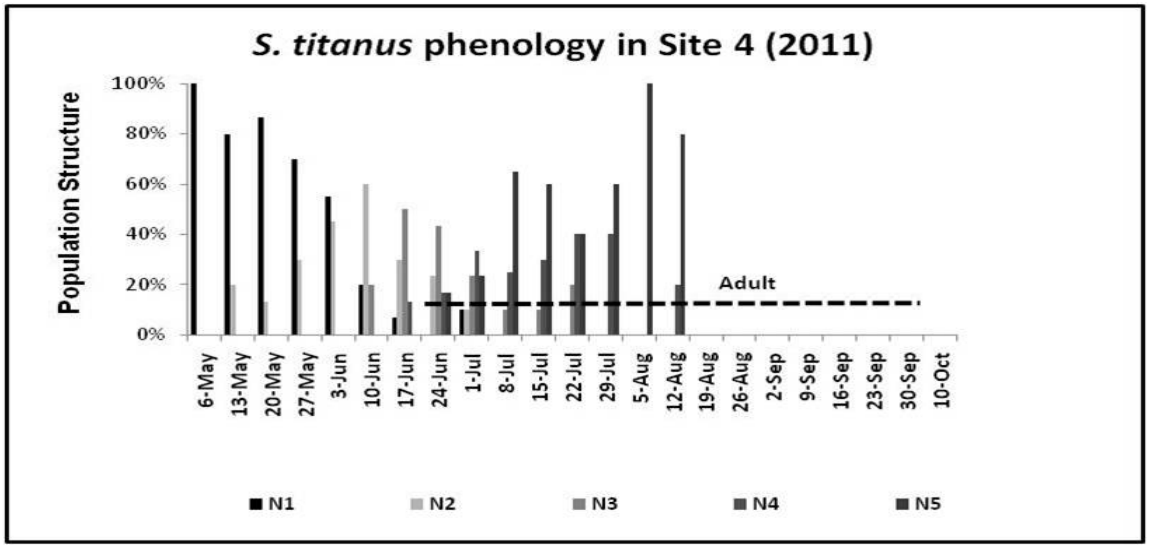

S. titanus phenology in Site 2 (2012)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
14-May
21-May
29-May
6-Jun
12-Jun
20-Jun
27-Jun
5-Jul
12-Jul
19-Jul
26-Jul
1-Aug
11-Aug
20-Aug
9-Sep
23-Sep
10-Oct
N1
N2
N3
N4
N5

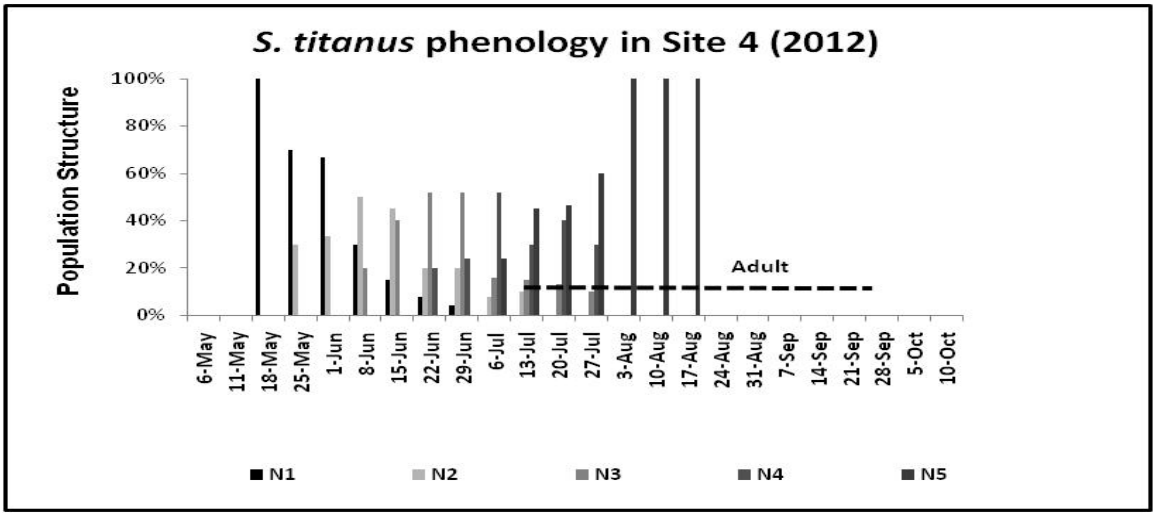

S. titanus phenology in Site 3 (2010)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
20-May
26-May
4-Jun
10-Jun
18-Jun
24-Jun
1-Jul
8-Jul
14-Jul
4-Aug
25-Aug
23-Sep
10-Oct
N1
N2
N3
N4
N5

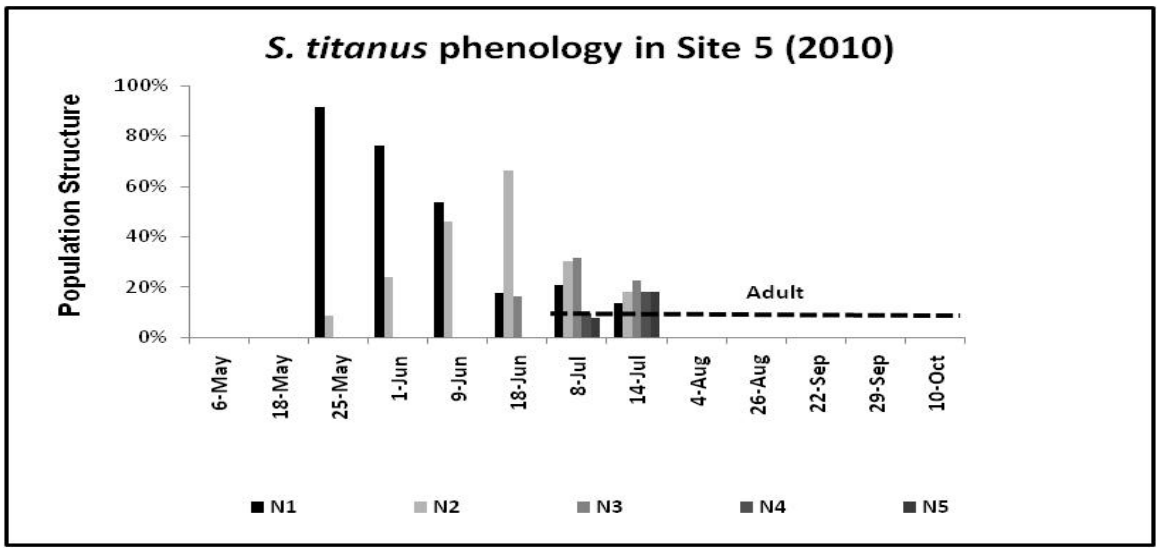

S. titanus phenology in Site 3 (2011)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
20-May
24-May
3-Jun
10-Jun
17-Jun
24-Jun
1-Jul
8-Jul
14-Jul
22-Jul
29-Jul
5-Aug
24-Aug
9-Sep
21-Sep
10-Oct
N1
N2
N3
N4
N5

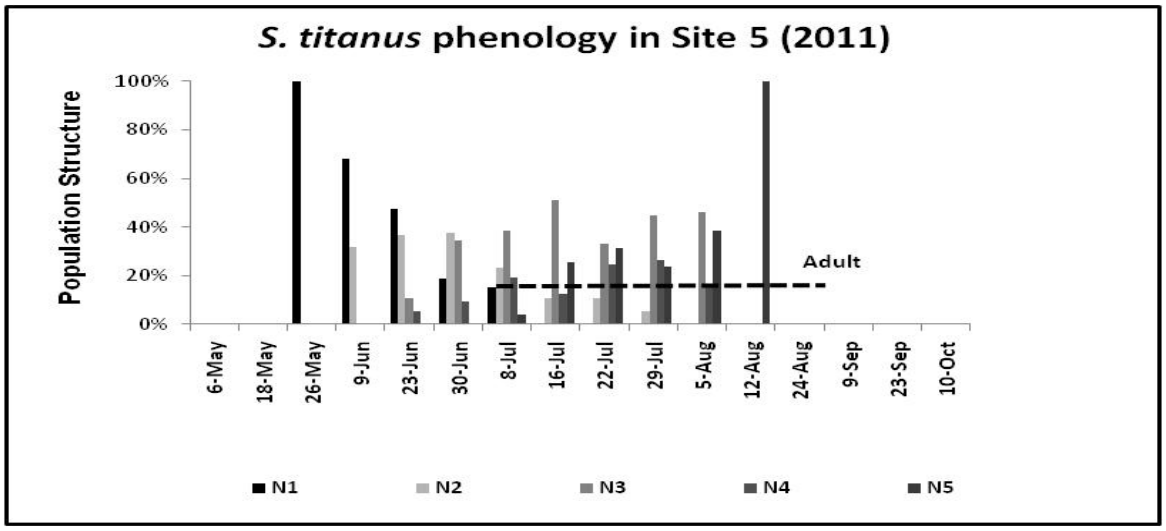

S. titanus phenology in Site 3 (2012)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
12-May
18-May
26-May
02-Jun
9-Jun
15-Jun
23-Jun
26-Jun
5-Jul
16-Jul
25-Jul
2-Aug
18-Aug
26-Aug
4-Sep
14-Sep
10-Oct
N1
N2
N3
N4
N5


S. titanus phenology in Site 4 (2010)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
14-May
21-May
28-May
4-Jun
11-Jun
18-Jun
25-Jun
2-Jul
9-Jul
16-Jul
23-Jul
30-Jul
6-Aug
20-Aug
27-Aug
3-Sep
10-Sep
17-Sep
24-Sep
1-Oct
10-Oct
N1
N2
N3
N4
N5


S. titanus phenology in Site 4 (2011)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
13-May
20-May
27-May
3-Jun
10-Jun
17-Jun
24-Jun
1-Jul
8-Jul
15-Jul
22-Jul
29-Jul
5-Aug
12-Aug
19-Aug
26-Aug
2-Sep
9-Sep
16-Sep
23-Sep
30-Sep
10-Oct
N1
N2
N3
N4
N5


S. titanus phenology in Site 4 (2012)
Population Structure
100%
80%
60%
40%
20%
0%
Adult
6-May
11-May
18-May
25-May
1-Jun
8-Jun
15-Jun
22-Jun
29-Jun
6-Jul
13-Jul
20-Jul
27-Jul
3-Aug
10-Aug
17-Aug
24-Aug
31-Aug
7-Sep
14-Sep
21-Sep
28-Sep
5-Oct
10-Oct
N1
N2
N3
N4
N5





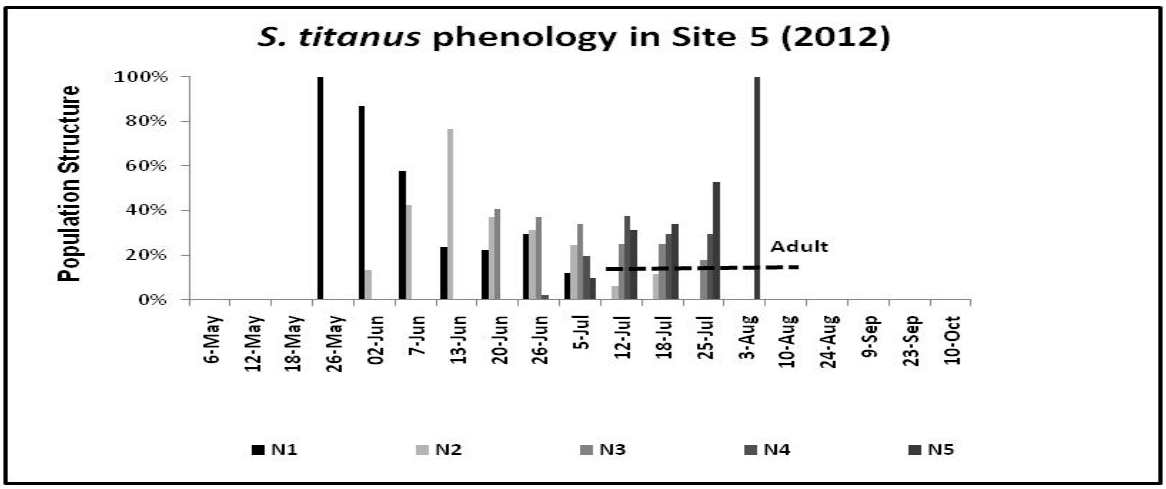
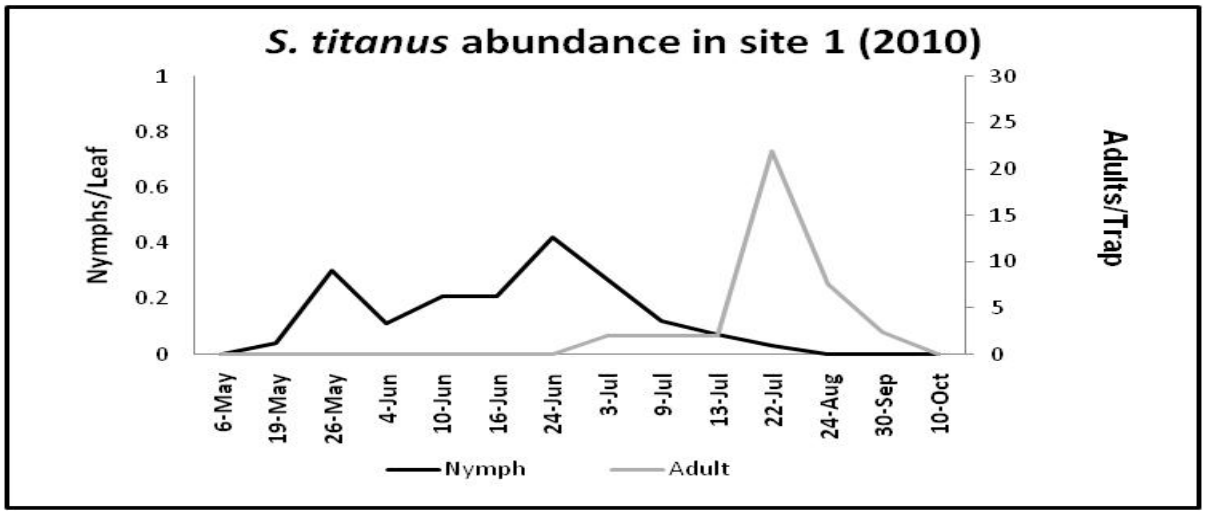
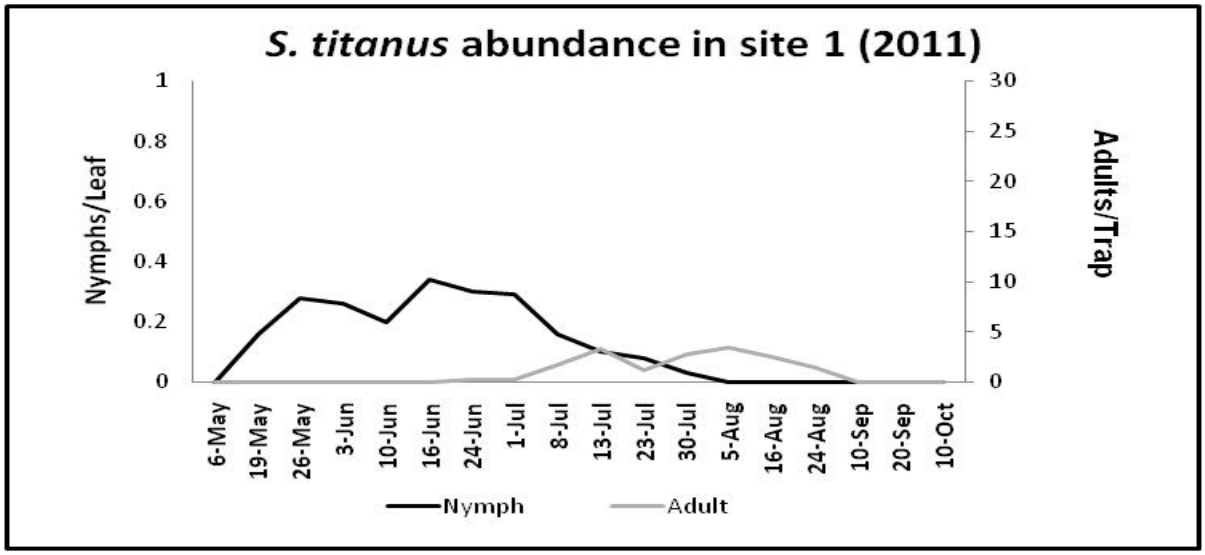


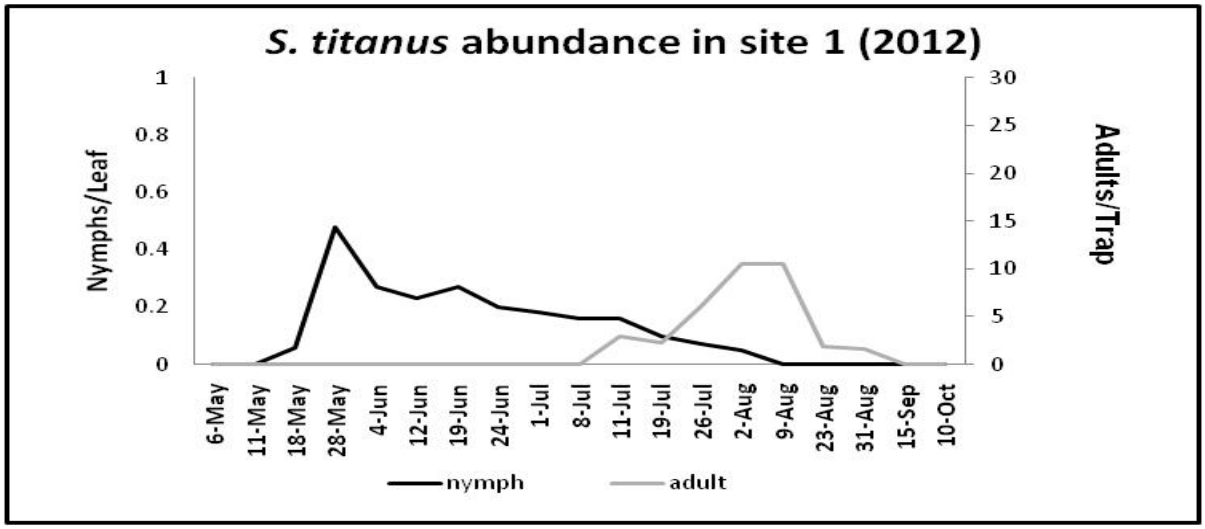
Figure 1- Shows the life cycle of *Scaphoideus titanus* in all the studied locations during 2010-2012.

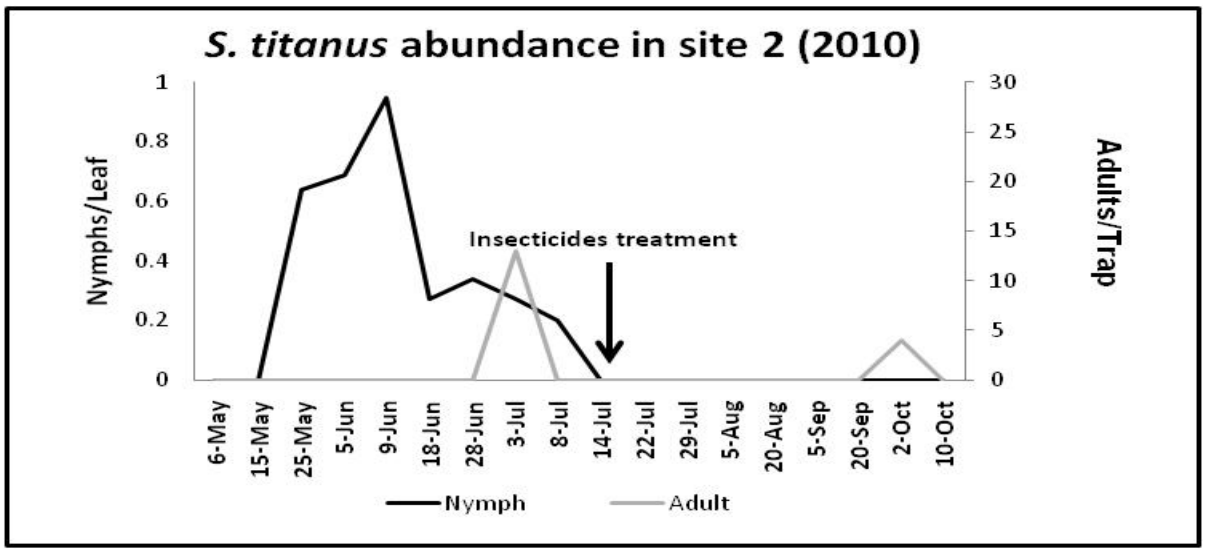
Leafhopper abundance showed large variation among the sites and the years (Figure 2). Nymph numbers appeared to be higher in site 2 compared to other sites for both 2010 and 2011, but not in 2012 where nymphs were more abundant in site 5. Sites 4 and 5 showed the lowest values in 2010 and 2011 respectively. Nymph peaks were observed in June except in 2012 when peaks were detected in May (site 1 and 2) and July (site 3, 4 and 5). Regarding adult populations, the highest captures during 2010 were seen in site 4, during 2011 in sites 2, 4 and 5, and during 2012 in site 4. Generally, the adult peaks were observed in July and early August. However, peaks were detected in late August in sites 1 (2010) and 5 (2010, 2011).

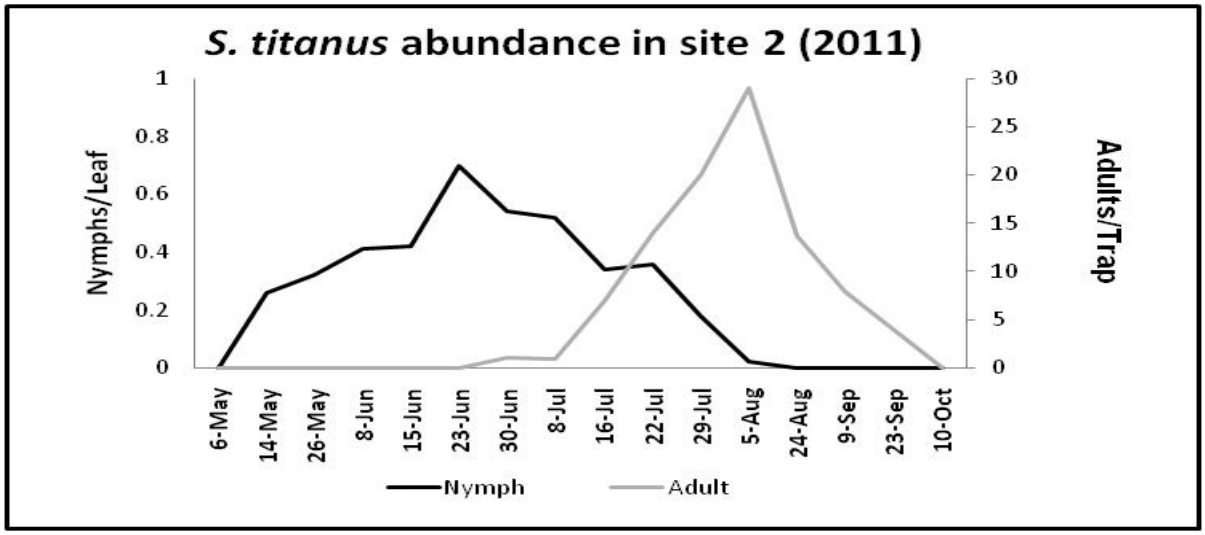
Regarding the temperature registered during the growing season in all the sites, April, May and September appeared to be warmer in 2011 (Figure 3). On the other hand, during the peak season for nymphs and adults (from June to August), 2012 registered the highest temperature among the other two years. The highest temperature during 2010 was in July, whilst August was the warmest in 2011 and 2012. The highest temperature for 2010 was registered in site 4 in July, site 4 also shows the highest temperature in 2011 in August. In 2012, the highest registered temperature was in August in site 1. On the contrary, site 5 was the coldest among all the experimental sites.

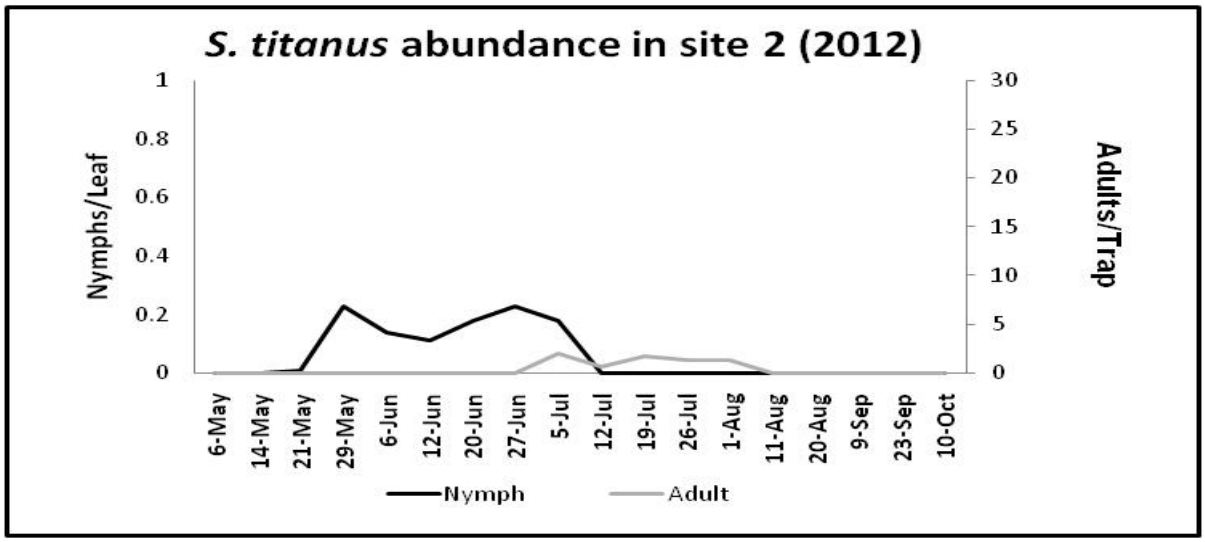

S. titanus abundance in site 1 (2010)
Nymphs/Leaf
Adults/Trap
1
0.8
0.6
0.4
0.2
0
30
25
20
15
10
5
0
6-May
19-May
26-May
4-Jun
10-Jun
16-Jun
24-Jun
3-Jul
9-Jul
13-Jul
22-Jul
24-Aug
30-Sep
10-Oct
Nymph
Adult

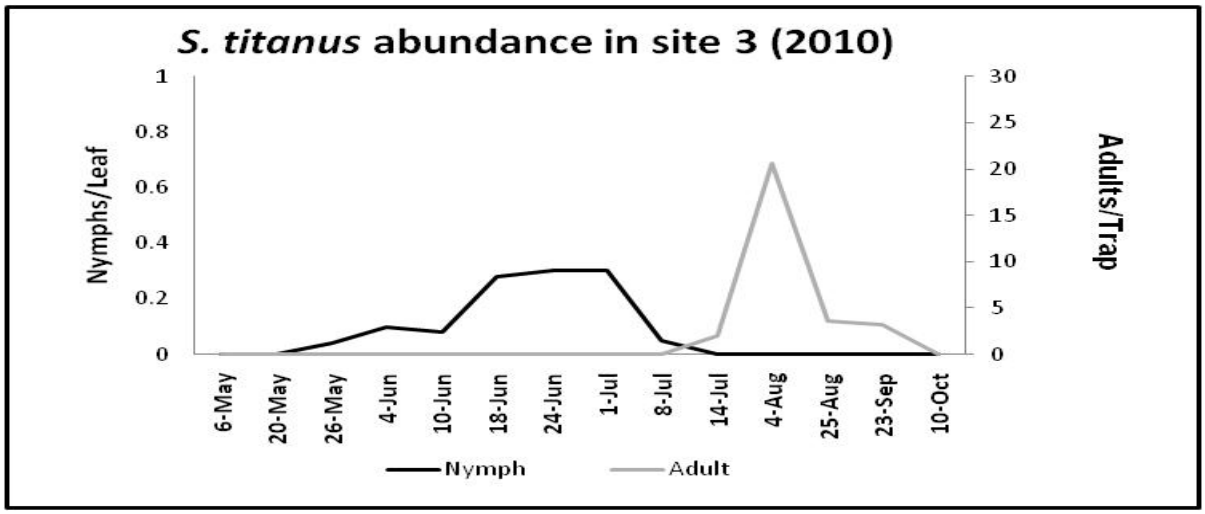

S. titanus abundance in site 1 (2011)
Nymphs/Leaf
Adults/Trap
1
0.8
0.6
0.4
0.2
0
30
25
20
15
10
5
0
6-May
19-May
26-May
3-Jun
10-Jun
16-Jun
24-Jun
1-Jul
8-Jul
13-Jul
23-Jul
30-Jul
5-Aug
16-Aug
24-Aug
10-Sep
20-Sep
10-Oct
Nymph
Adult

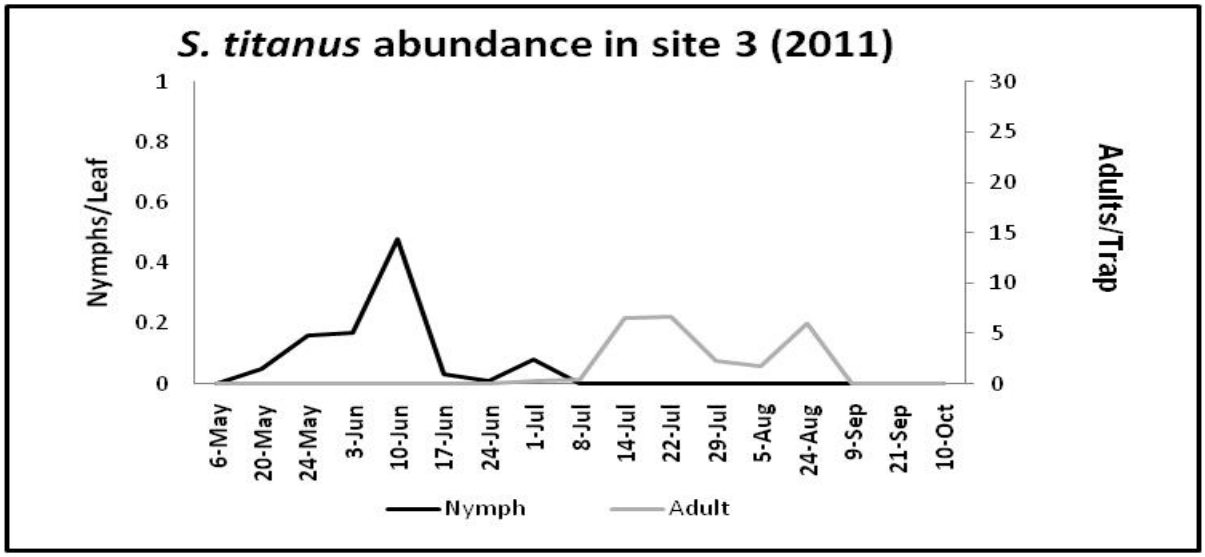

S. titanus abundance in site 1 (2012)
Nymphs/Leaf
Adults/Trap
1
0.8
0.6
0.4
0.2
0
30
25
20
15
10
5
0
6-May
11-May
18-May
28-May
4-Jun
12-Jun
19-Jun
24-Jun
1-Jul
8-Jul
11-Jul
19-Jul
26-Jul
2-Aug
9-Aug
23-Aug
31-Aug
15-Sep
10-Oct
nymph
adult

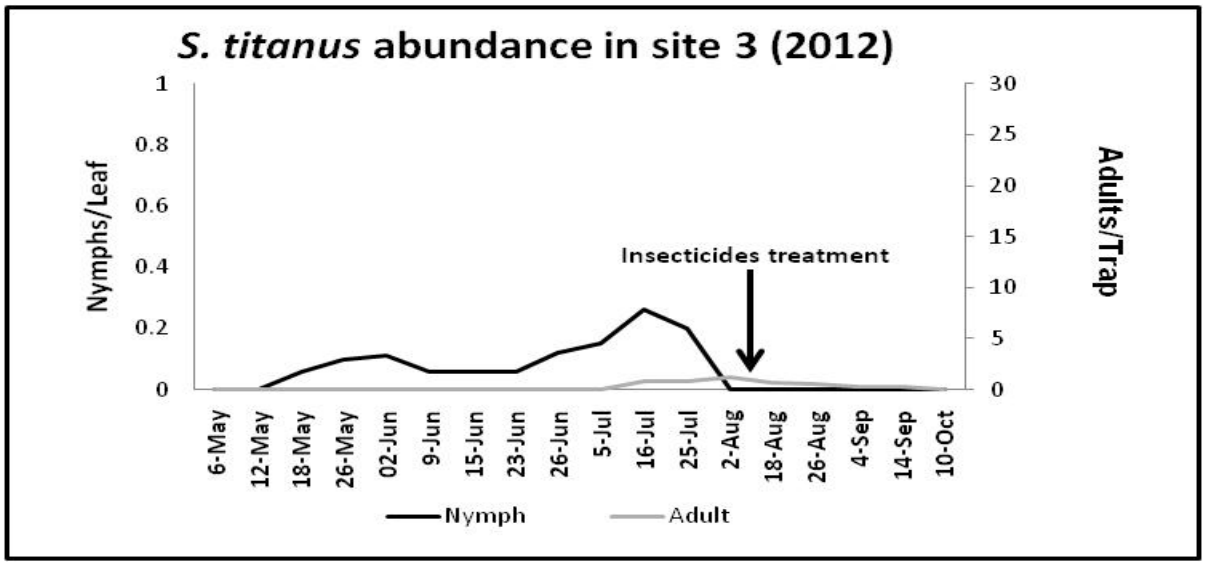

S. titanus abundance in site 2 (2010)
Nymphs/Leaf
Adults/Trap
Insecticides treatment
6-May
15-May
25-May
5-Jun
9-Jun
18-Jun
28-Jun
3-Jul
8-Jul
14-Jul
22-Jul
29-Jul
5-Aug
20-Aug
5-Sep
20-Sep
2-Oct
10-Oct
Nymph
Adult

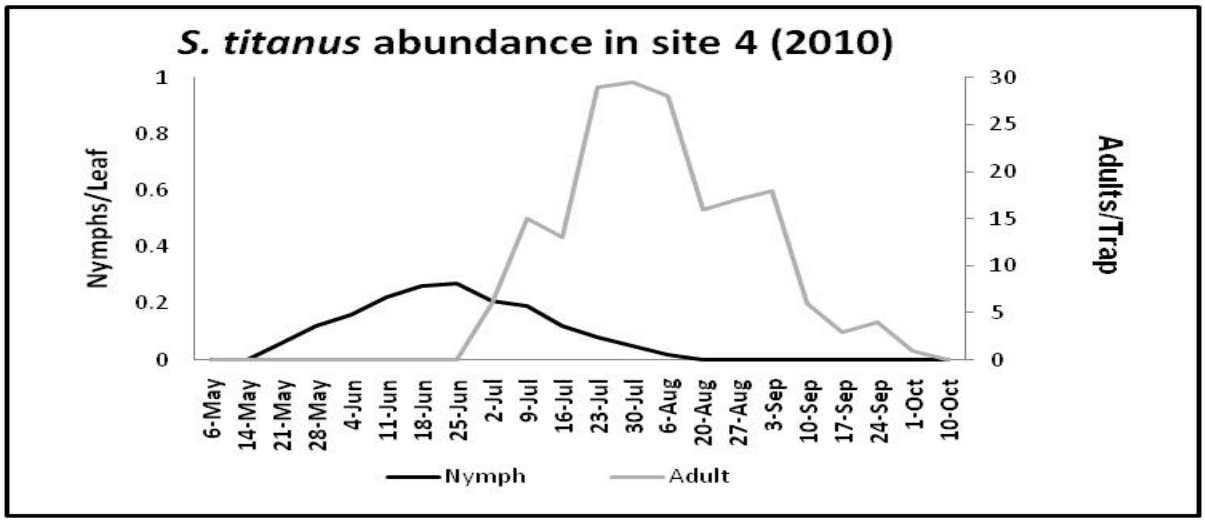

S. titanus abundance in site 2 (2011)
Nymphs/Leaf
Adults/Trap
6-May
14-May
26-May
8-Jun
15-Jun
23-Jun
30-Jun
8-Jul
16-Jul
22-Jul
29-Jul
5-Aug
24-Aug
9-Sep
23-Sep
10-Oct
Nymph
Adult

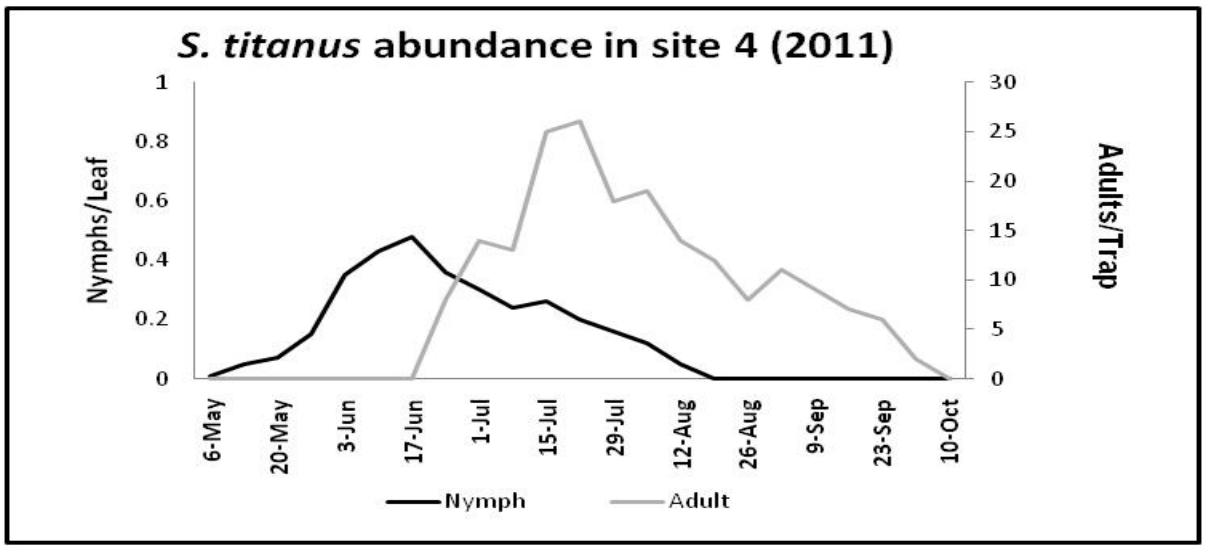

S. titanus abundance in site 2 (2012)
Nymphs/Leaf
Adults/Trap
6-May
14-May
21-May
29-May
6-Jun
12-Jun
20-Jun
27-Jun
5-Jul
12-Jul
19-Jul
26-Jul
1-Aug
11-Aug
20-Aug
9-Sep
23-Sep
10-Oct
Nymph
Adult

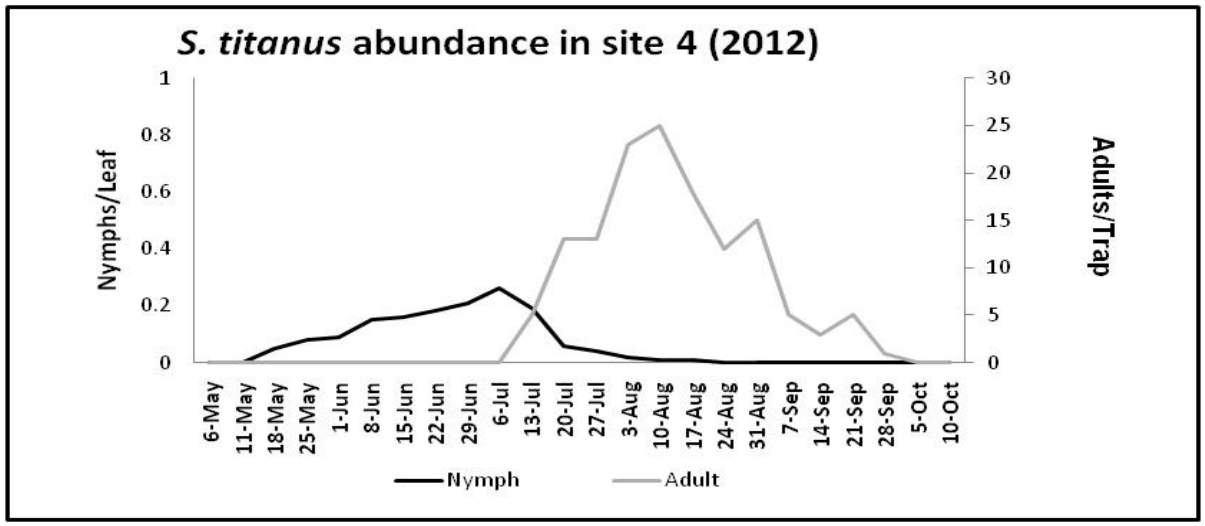

S. titanus abundance in site 3 (2010)
Nymphs/Leaf
Adults/Trap
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
6-May
20-May
26-May
4-Jun
10-Jun
18-Jun
24-Jun
1-Jul
8-Jul
14-Jul
4-Aug
25-Aug
23-Sep
10-Oct
Nymph
Adult

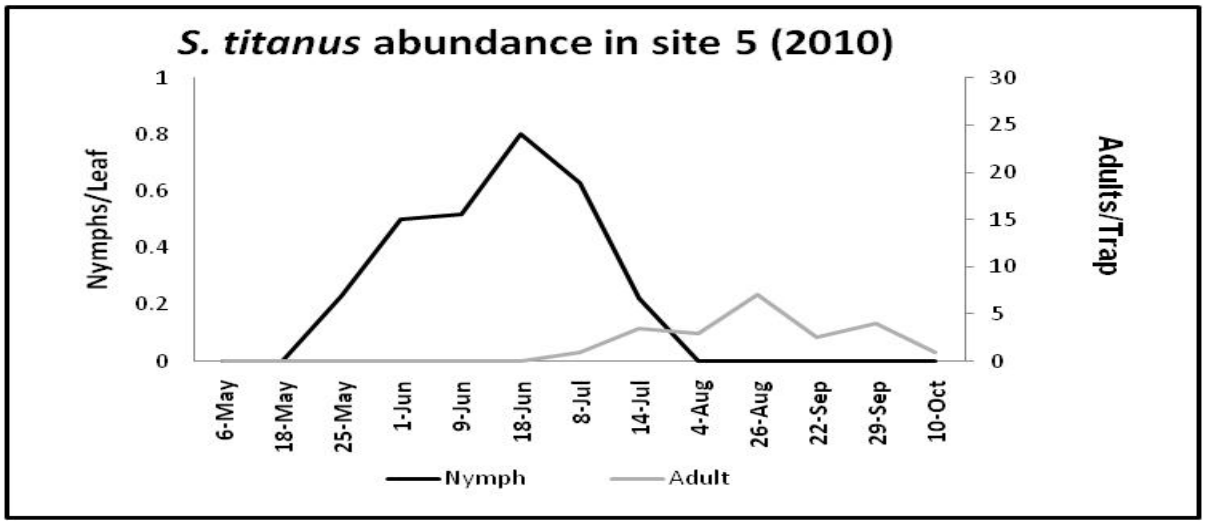

S. titanus abundance in site 3 (2011)
Nymphs/Leaf
Adults/Trap
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
6-May
20-May
24-May
3-Jun
10-Jun
17-Jun
24-Jun
1-Jul
8-Jul
14-Jul
22-Jul
29-Jul
5-Aug
24-Aug
9-Sep
21-Sep
10-Oct
Nymph
Adult

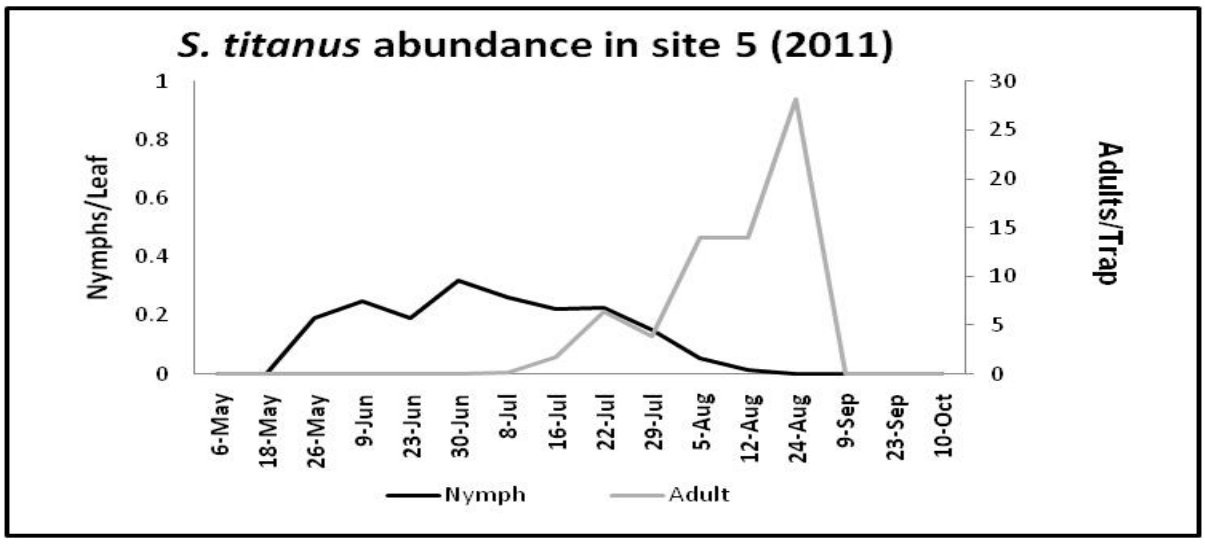

S. titanus abundance in site 3 (2012)
Nymphs/Leaf
Adults/Trap
Insecticides treatment
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
6-May
12-May
18-May
26-May
02-Jun
9-Jun
15-Jun
23-Jun
26-Jun
5-Jul
16-Jul
25-Jul
2-Aug
18-Aug
26-Aug
4-Sep
14-Sep
10-Oct
Nymph
Adult


S. titanus abundance in site 4 (2010)
Nymphs/Leaf
Adults/Trap
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
6-May
14-May
21-May
28-May
4-Jun
11-Jun
18-Jun
25-Jun
2-Jul
9-Jul
16-Jul
23-Jul
30-Jul
6-Aug
20-Aug
27-Aug
3-Sep
10-Sep
17-Sep
24-Sep
1-Oct
10-Oct
Nymph
Adult


S. titanus abundance in site 4 (2011)
Nymphs/Leaf
Adults/Trap
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
6-May
20-May
3-Jun
17-Jun
1-Jul
15-Jul
29-Jul
12-Aug
26-Aug
9-Sep
23-Sep
10-Oct
Nymph
Adult


S. titanus abundance in site 4 (2012)
Nymphs/Leaf
Adults/Trap
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
6-May
11-May
18-May
25-May
1-Jun
8-Jun
15-Jun
22-Jun
29-Jun
6-Jul
13-Jul
20-Jul
27-Jul
3-Aug
10-Aug
17-Aug
24-Aug
31-Aug
7-Sep
14-Sep
21-Sep
28-Sep
5-Oct
10-Oct
Nymph
Adult


S. titanus abundance in site 5 (2010)
Nymphs/Leaf
Adults/Trap
1
0.8
0.6
0.4
0.2
0
30
25
20
15
10
5
0
6-May
18-May
25-May
1-Jun
9-Jun
18-Jun
8-Jul
14-Jul
4-Aug
26-Aug
22-Sep
29-Sep
10-Oct
Nymph
Adult


S. titanus abundance in site 5 (2011)
Nymphs/Leaf
Adults/Trap
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0.8
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6-May
18-May
26-May
9-Jun
23-Jun
30-Jun
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16-Jul
22-Jul
29-Jul
5-Aug
12-Aug
24-Aug
9-Sep
23-Sep
10-Oct
Nymph
Adult

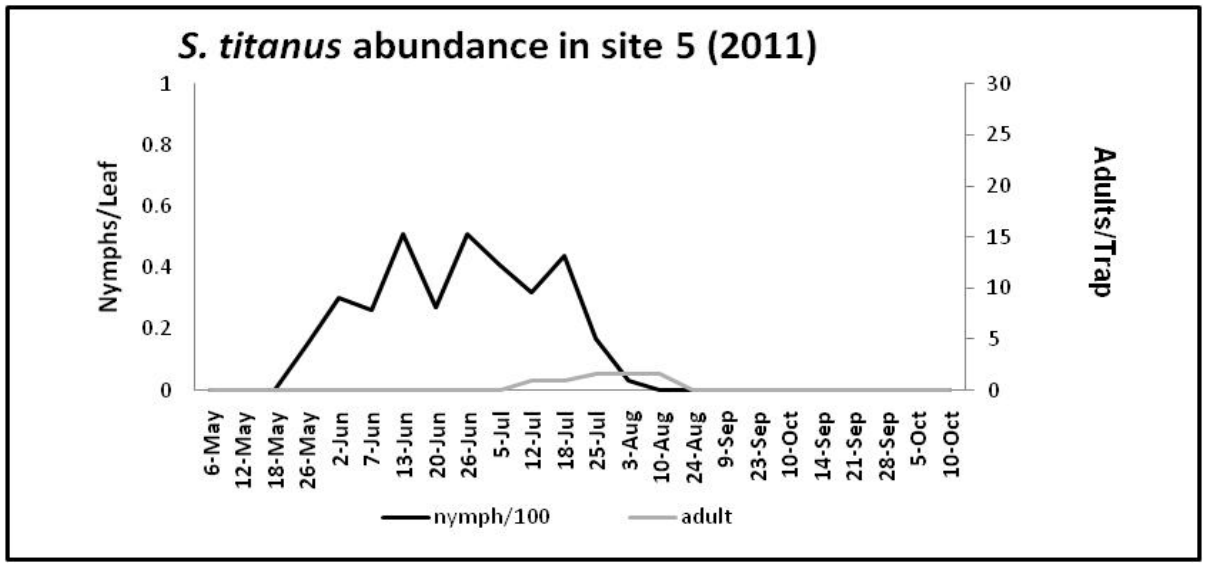
S. titanus abundance in site 5 (2011)
Nymphs/Leaf
Adults/Trap
1
0.8
0.6
0.4
0.2
0
30
25
20
15
10
5
0
6-May
12-May
18-May
26-May
2-Jun
7-Jun
13-Jun
20-Jun
26-Jun
5-Jul
12-Jul
18-Jul
25-Jul
3-Aug
10-Aug
24-Aug
9-Sep
23-Sep
10-Oct
14-Sep
21-Sep
28-Sep
5-Oct
10-Oct
nymph/100
adult

Figure 2- *Scaphoideus titanus* abundance in all the sites for 2010-2012

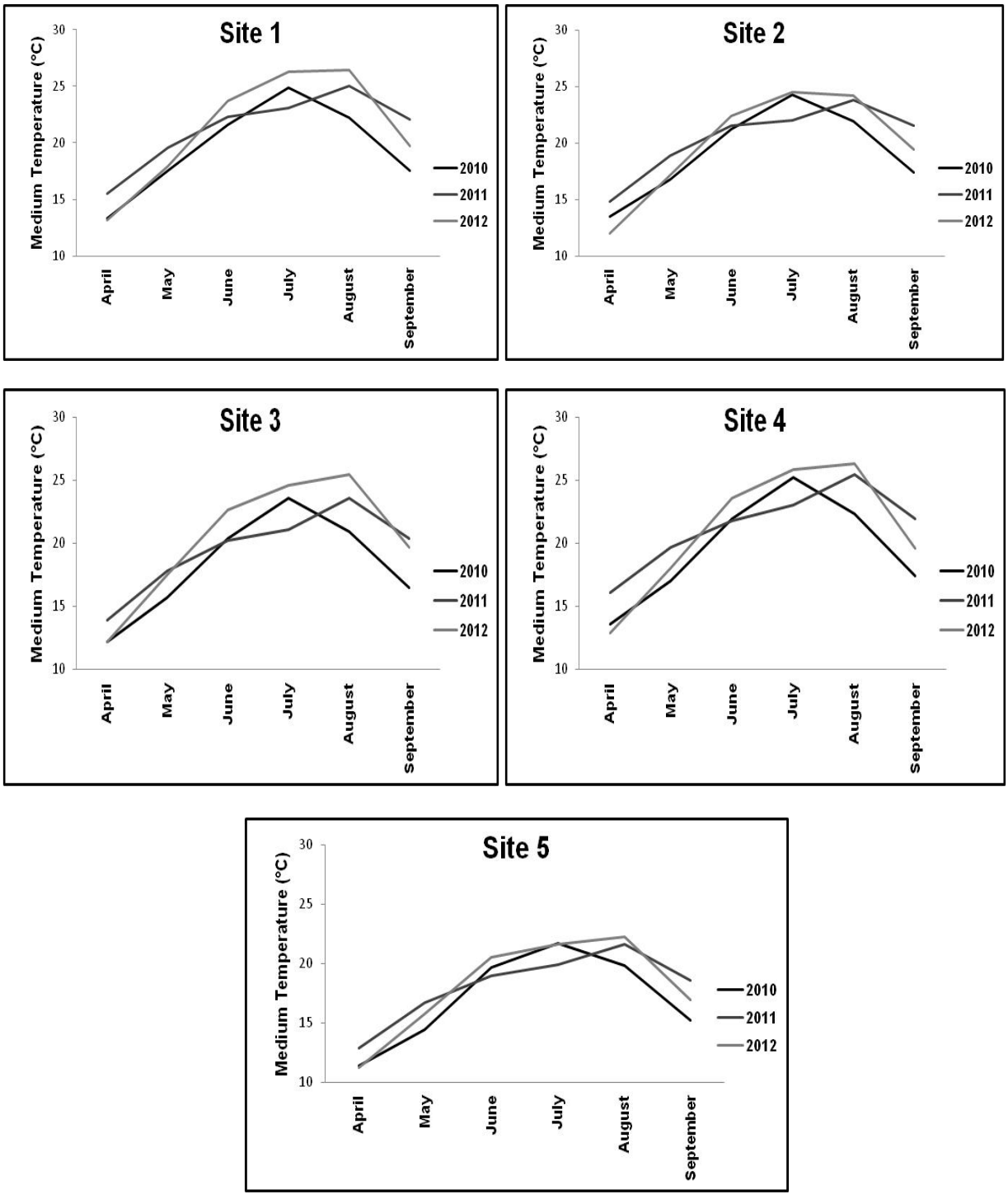

Figure 3- The temperature during the growing season in all the sites

Relation between appearance dates and temperature

The first appearance of nymphs for the five locations (2010-2012) was noticed within the range of 280-430 DD. In fact, the first nymph appearance in the majority of the experimental sites took place within 300 DD. Actually, site 2 needed the lowest DD in 2010 and 2012 compared to other locations. Whereas, the range of cumulate temperature (DD>8.7°C) for the first adult appearance was wider in the five locations (700-1200). Likewise, the 50% cumulate nymph population in all the locations happened within values of 400-800 DD. For 50% cumulate adult population, the range was 1000-1400. Figure 4 shows the regression between the averages of medium temperature from January till the first appearance of nymphs and adults, and with the cumulated 50% of the nymph and adult populations. This regression was significant only for the first appearance of the nymphs.

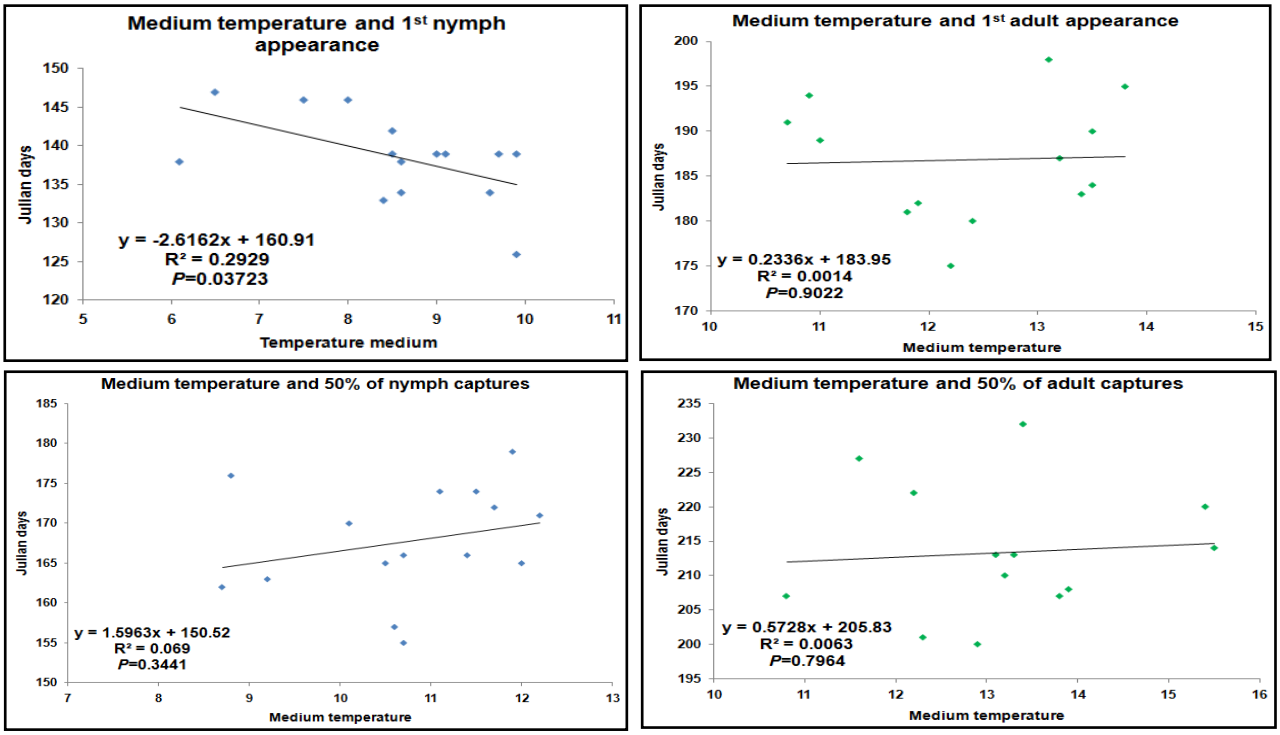

Figure 4- Regression between medium temperature and the first appearance of nymphs and adults, and with the 50% of nymphs and adults captures

The regression between the averages of maximum temperature from January till the first appearance of nymphs and adults, and with the cumulated nymph and adult populations is shown in figure 5. Similarly to before, the only significant regression was found with the first nymph appearance.

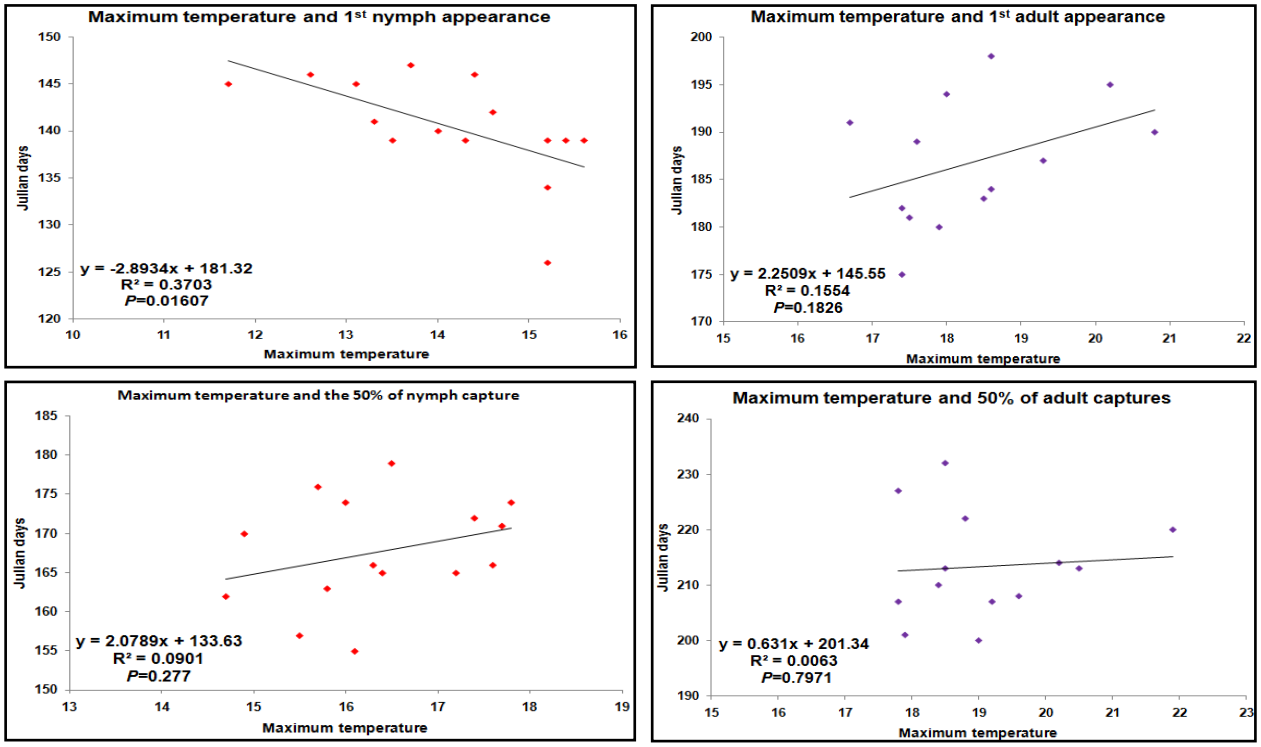

Figure 5- Regression between maximum temperature and the first appearance of nymphs and adults, and with the 50% of nymphs and adults captures

Discussion

Regarding *S. titanus* phenology, data confirm previous observations on the life cycle of this leafhopper in Veneto region (Belli, 1984; Pavan *et al*., 1987; Posenato and Girolami, 1994; Posenato, 2001) and add information on the potential variation among locations different in altitude, exposure and climatic conditions. The appearance dates of *S. titanus* nymphs and adults in Veneto can be compared with those reported for other Italian regions and countries (Table 3).

Table 3- Appearance dates of nymphs and adults in some regions or countries where *Scaphoideus titanus* is widely spread.

Country / Region	Nymph appearance date	Adult appearance date
Italy / Liguria (Vidano, 1966)	2nd half of May	First decade of July
Italy / Piedmont (Bosco *et al*., 1997)	Not mentioned	Mid/end of July
Italy / Liguria and Tuscany (Mazzoni *et al*., 2001)	Mid-May	Beginning of June
Italy / Trentino (Dal Rì and Capra, 2003)	21 May	19 July
France / Armagnac (Magarey, 1986)	3rd week of May	4th week of July
France / Corse (Bagard, 1987)	10-25 of May	End of July
France / Bordeaux (Decante and Van Helden, 2006)	End of April - beginning of May	End of July
Hungary (Dèr *et al*., 2007)	Not mentioned	5 July
Serbia (Krnjajić *et al*., 2007)	Mid-May	Third decade of June
Austria (Steffek *et al*., 2007)	Not mentioned	Beginning of June - late September
Switzerland (Gugerli, 2007)	Mid-May	Late July - early September

In our study the leafhopper phenology showed variation among sites and years. In 2010, the first nymphs appeared from 19 to 26 May, and the first adults from 2 to 14 July. In the warmest year (2011), the first nymphs appeared from 6 to 26 May and the first adults from 24 June to 8 July. Finally in 2012, nymphs appeared from 18 to 26 May and adults from 5 to 16 July. At the same time it is interesting to note that site 3 showed a delay in leafhopper phenology despite it was not the coldest one. If climatic factors (in particular temperature) varied among years and locations affecting appearance dates, other factors seem to influence *S. titanus* phenology. The effects of

temperature were clear on nymph appearance dates. Medium and maximum temperatures significantly affected nymph appearance and in particular, the latter was earlier with increasing temperature. In contrast, there were no significant relationships between temperature and cumulated leafhopper populations.

Our results confirm previous investigation on hatching dynamics of *S. titanus* (Chuche and Thiéry, 2009). Warm temperature was associated to early detection of first nymph appearance. Moreover our results stress that late winter, spring temperature are determinant for the emergence of nymphs, while no clear association exist of temperature with immature development.

The importance of understanding the relation between temperature and insect population is recently increasing because it could help in predicting the development of *S. titanus*, thus knowing the best time to apply pest management practices. Some studies suggest that using the Degree Days above the threshold of 8.7°C can help viticulture growers for a more accurate monitoring to pests. In that case, farmers would achieve a better protection for their fields from *S. titanus* and the disease it transmits. The study of Rigamonti *et al*. (2011) is an example of similar studies that used the monitoring information and the relation with temperature for the development of a temperature-driven phonological model to stimulate the occurrence time of *S. titanus* nymphs in the vineyards. This study took place in Switzerland, but the model could be developed for other regions.

The phenological data presented at this work could be useful to build up a forecasting model for the prediction of *S. titanus* occurrence, particularly in new colonization regions. This means that technicians may be provided with an updated phenological data to determine the best timing for the mandatory pesticides treatments. So, a continuous research on *S. titanus* phenology in different regions together with further investigation of the relation with the field temperature under the challenge of climate change, is strongly needed to improve and develop the capability of the forecasting models.

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Chapter 5

Scaphoideus titanus (Hemiptera: Cicadellidae) response to grape Cultivars and its ability to distinguish among stimuli emitted from healthy or phytoplasma infected leaves

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Haya AbouAssaf collected part of the data, contributed to their analysis and drafted the manuscript. This work was made in cooperation with Dr. Alberto Pozzebon, Dr. Nicola Mori and Prof. Carlo Duso.

Abstract

The leafhopper *Scaphoideus titanus* is the vector of Flavescence dorée (FD), a serious disease in European vineyards. The preference of this leafhopper for different grape Cultivars and the role of stimuli in host recognition are not well known despite their potential implications for pest control. In this study, the attractiveness of four grape Cultivars to different life stages of *S. titanus* was evaluated. Furthermore, shoots infected by Flavescence dorée (FD) or Bois noir (BN) or not infected were used to investigate the ability of this leafhopper to distinguish among different stimuli. The results of this study showed different levels of preference of *S. titanus* towards the investigated Cultivars. This preference varied according to the leafhopper stage. Moreover, *S. titanus* was found to be able to distinguish between stimuli emitted by healthy or disease infected shoots, while no preference was exhibited towards stimuli emitted by shoots infected by FD or BN phytoplasmas. These results could contribute in understanding *S. titanus* behaviour and its distribution pattern within vineyards. Hence, they could contribute to the development of tools for the monitoring and control strategy.

Introduction

The grapevine leafhopper *Scaphoideus titanus* Ball is a phloem-feeding species belonging to the family Cicadellidae (Schvester *et al*., 1962; 1969; Bertin *et al*., 2007). This leafhopper is the vector of *Candidatus* Phytoplasma vitis 16SrV, the causal agent of the disease Flavescence Dorée (FD), one of the most threatening among the Grapevine Yellow (GY) diseases in Europe (Boudon-Padieu, 2003). *Scaphoideus titanus* appears unable to transmit the agent of another GY, the Bois noir (BN) *Candidatus* Phytoplasma solani 16SrXII (Carraro *et al*., 1994; Martini *et al*., 1999; Mori *et al*., 2002), a major disease of grapevine in Italy (Bondavalli *et al*., 2005; Borgo *et al*., 2005; Duso *et al*., 2010).

In Europe, *S. titanus* feeds on cultivated grapes (*Vitis vinifera* L.). It has one generation per year and overwinters as eggs usually laid under two years-old bark (Vidano, 1966; Decante and Helden, 2006). Nymphs usually are found on leaf undersurfaces and are not able to move for long distances. Adults can fly but they also do not move away from their host plants (Lessio and Alma, 2004).

Scaphoideus titanus, originating from North America (Barnett, 1976), was probably introduced to Europe as overwintering eggs (Vidano, 1966; Boudon-Padieu, 1999). It was reported in southern France in 1958 (Bonfils and Schvester, 1960), then it spread to Italy where it was reported for the first time in Liguria (Vidano, 1966). Nowadays, this leafhopper is spread in various Italian regions (Alma, 2002; Viggiani, 2002; 2004; Sancassani *et al*., 2008).

Scaphoideus titanus acquires FD agent by feeding on infected plants and then transmits it to other plants when feeding on them (Conti and Vidano, 1988; Bressan *et al*., 2006). All the feeding stages of *S. titanus* are able to acquire the FD phytoplasma but a latent period is needed for the phytoplasma to multiply and achieve persistency in the vector (Conti and Vidano, 1988; Weintraub and Beanland, 2006; Bressan *et al*., 2006).

Scaphoideus titanus has been the subject of many studies to understand the acquisition and transition way of FD phytoplasma (Schvester *et al*., 1969; Alma *et al*., 1997; Bressan *et al*., 2005; Weintraub and Beanland, 2006) rather than investigating its biology and behaviour (Lessio and Alma, 2004; Rigamonti *et al*., 2011).

Grapevine Cultivars have an important effect on the incidence of infected leafhoppers, consequently they have an influence on the spread of FD disease (Bressan *et al*., 2005). The susceptibility of grapevine Cultivars to FD varies greatly. In Italy, two molecular types of FD were distinguished and they are associated to some "preference" for different Cultivars (Martini *et al*., 1999; Bertaccini *et al.,* 2000; Angelini *et al*., 2001; Mori *et al*., 2002). The Cultivars Chardonnay, Pinot Noir, Pinot Gris, Cabernet Franc, Cabernet Sauvignon, Barbera, Sangiovese, Soave, and Glera (Prosecco) are more susceptible to FD than Merlot, Sauvignon Blanc and Syrah (Pavan *et al*., 1997; Belli *et al.*, 2000; Vercesi and Scattini, 2000; Bellomo *et al*., 2007; Belli *et al*., 2010). Unfortunately, the attractiveness of grapevine Cultivars towards *S. titanus* is not well studied. It is not known if Cultivars susceptible to FD or BN are preferred or not by this vector. This data could be helpful for effective pest control programs.

In this framework, little attention was paid on *S. titanus* attraction to stimuli emitted by plants, and the role of plant volatiles in host detection (Mazzoni *et al.*, 2009). These authors found that olfactory cues may play a role in host plant detection by *S. titanus* nymphs. Nymphs were able to distinguish among volatile compounds emitted by various grapevine organs. On the other hand, vision abilities may interact with host plant stimuli in host detection by leafhoppers (Todd *et al.*,

1990). So depending on stimuli perception and vision *S. titanus* is able to detect preferred host plants, but its ability to distinguish between healthy and infected plants based on the respective stimuli has not been studied.

In this study, the preference of *S. titanus* for four grapevine Cultivars and its ability to perceive stimuli emitted from healthy or disease infected grapevine shoots has been investigated.

Materials and methods

This research was carried out in 2011 and 2012. Nymphs and adults of *S. titanus* were collected from an experimental vineyard located at the University of Padova (Department DAFNAE, Legnaro, Italy) using a vacuum. Experiments were carried out in screen house and laboratories located at Department DAFNAE. In the first study (Cultivar effects) we distinguished between "young nymphs" (1^{st} and the 2^{nd} instars together) and "nymphs" (3^{rd}, 4^{th} and 5^{th} instars altogether) while in the second (detection of stimuli from healthy or infected shoots) five nymph-instars were used.

The response by *Scaphoideus titanus* to grapevine Cultivars:

Experimental design

Four grapevine Cultivars of economic importance with a different susceptibility to FD were selected: Chardonnay, Cabernet franc, Merlot and Glera (Prosecco). Seedlings belonging to these Cultivars were used as potted plants bearing four to five leaves. Four potted plants were placed inside a cage, each plant from one Cultivar. The plants were placed in the four corners of cages in an experimental screen house. A Petri dish with 20 individuals of *S. titanus* was placed in the centre of the cage to guarantee an equal distance from the four potted plants (figure 1). Eight cages were used. The experiment was repeated twice with each life stage of *S. titanus* and each time with new set of plants. After the release of 20 individuals, the cages were checked after one (h1), six (h6), twelve (h12) and twenty four (h24) hours to assess the number of leafhoppers on each Cultivar over the time.

Figure 1- The experimental design showing the distribution of the four Cultivars and the Petri dish in the centre.

Statistical analysis

Data were analysed using a repeated REML (Restricted Maximum Likelihood) analysis of variance using the PROC MIXED (SAS Institute, 1999). The proportion (number of insect found on a specific Cultivar / total number of insect found in a cage) of insects found on each Cultivar during each assessment time was considered as dependent variable. In modelling, Cultivar, time of assessment and their interaction were considered as source of variation and their effect was evaluated using F test ($\alpha = 0.05$). Using the LSMEAN statement we performed a t- test on the pairwise comparison of different Cultivars ($\alpha = 0.05$). An angular transformation was applied to data to meet the assumption of REML analysis of variance.

Scaphoideus titanus perception of stimuli emitted by healthy and infected shoots:

Plant material

Chardonnay grapevine shoots having small leaves were used as stimuli source. The FD infected shoots were collected from symptomatic plants in vineyards (located in Treviso and Vicenza districts, in 2011 and 2012 respectively) where only FD was widespread among GYs. The BN

infected shoots were collected from symptomatic plants in a vineyard located in Verona district where only BN was present among GYs. The healthy shoots were collected from a vineyard located in Verona district where no GY symptomatic plants were observed. The presence/ absence of the phytoplasmas in plants and in shoots were tested and certified by molecular analyses performed by CRA-VIT (Centro di ricerca per la Viticoltura, Conegliano TV).

Olfactometer used in the study

The experiment was conducted with a vertical glass Y-olfactometer (the stem and the arms are 12.0 cm length, and 4.3 cm width; figure 2). The two arms were connected to glass flasks in which the shoots were placed. The olfactometer was located on a black board, and the two glass flasks were covered with white sheets to guarantee that the decision of the insect was made by the stimuli only not by interaction with the vision ability. The insects were tested individually, by inserting each insect into the main stem of the olfactometer. After that the time needed for the insect to make its decision was recorded. To be considered as a decision each individual had to reach beyond the half of the arm. When the individual took more than 15 minutes to make a decision or just it moved back and forward in the stem, it was considered as "No-choice". After five insects, the glass flasks were reversed, and after ten the olfactometer was cleaned by Acetone.

Figure 2- The vertical glass Y-olfactometer used in the experiments

Statistical analysis

The χ^2 test ($\alpha = 0.05$) for homogeneity of proportion was used to test whether the proportion of insects was the same for healthy or infected shoots. Considering time needed to make the decision (expressed in seconds) as the response, F test ($\alpha = 0.05$) was used to study the effect of age (nymphs or adults), decision (the No-choice option was excluded for this part) and their interaction. R software release 2.14.0 (R Development Core Team, 2011) was used in statistical analysis.

Results

The response by *Scaphoideus titanus* to grape Cultivars

A significant variation in the proportion of young nymphs was found among Cultivars ($F_{3, 239} = 4.21$; $P=0.0063$). No effect of time ($F_{3, 239}=0.08$; $P=0.971$), nor of the interaction time*Cultivar was found ($F_{9, 239}=0.48$; $P=0.888$). The proportion of insects found on Cabernet franc and Chardonnay Cultivars was similar ($t_{239}=0.11$; $P=0.915$; Figure 3), and the proportion of insects found on these Cultivars was higher compared to those reported for Merlot (vs. Chardonnay: $t_{239}=2.85$; $P=0.005$; vs. Cabernet franc: $t_{239}=2.74$; $P=0.007$; Figure 3) and Glera (vs. Chardonnay: $t_{239}=2.20$; $P=0.029$; vs. Cabernet franc: $t_{239}=2.09$; $P=0.038$; Figure 3). No differences were observed between Merlot and Glera ($t_{239}=0.65$; $P= 0.516$; Figure 3).

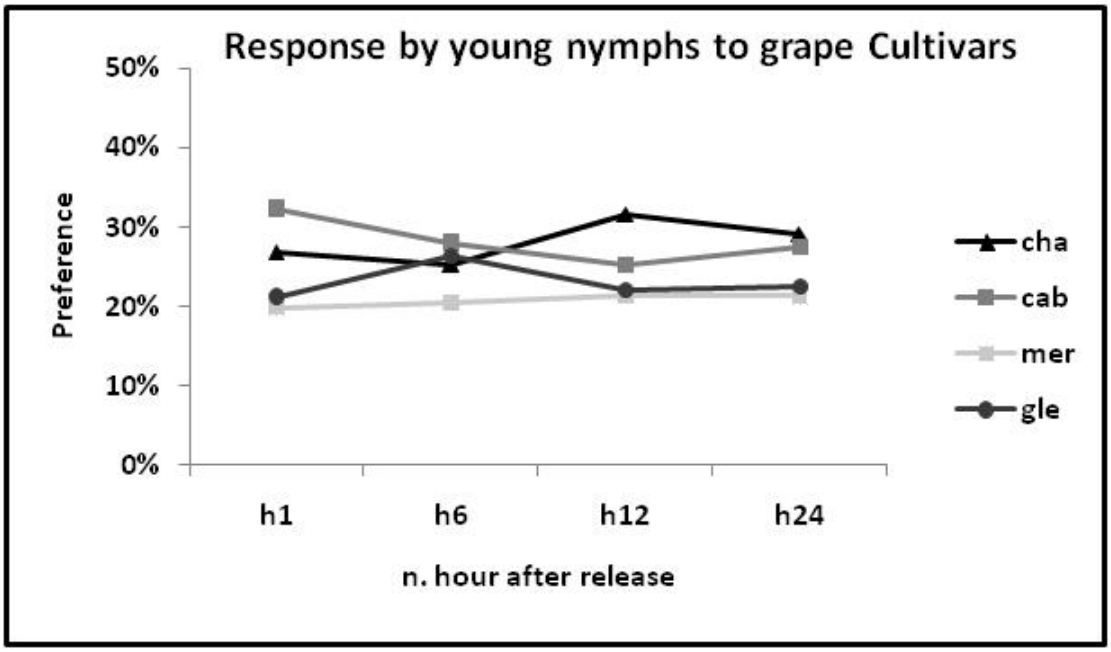

Figure 3- Proportion of young nymphs of *Scaphoideus titanus* on observed on different Cultivars during the experimental period (h1: one hour after release; h6: six hours after release; h12: twelve hours after release; h24: twenty four hours after release). cha: Chardonnay; cab: Cabernet franc; mer: Merlot; gle: Glera.

A significant variation in the proportion of nymphs was found among the Cultivars ($F_{3, 239}$=2.88; P=0.037). No effect of time ($F_{3, 239}$=0.03; P=0.994) nor of the interaction time*Cultivar was found ($F_{9, 239}$=0.47; P=0.893). The proportion of nymphs found on Chardonnay, Glera and Merlot was similar (Chardonnay vs. Glera: t_{239}=0.26; P=0.796; vs. Merlot: t_{239}=0.31; P=0.760; Glera vs. Merlot: t_{239}=0.05; P=0.963; figure 4). The proportion of nymphs found on those three Cultivars was higher compared to that found on Cabernet franc (vs. Chardonnay: t_{239}=2.57; P=0.011; vs. Glera: t_{239}=2.31; P=0.022; vs. Merlot: t_{239}=2.27; P=0.024; figure 4).

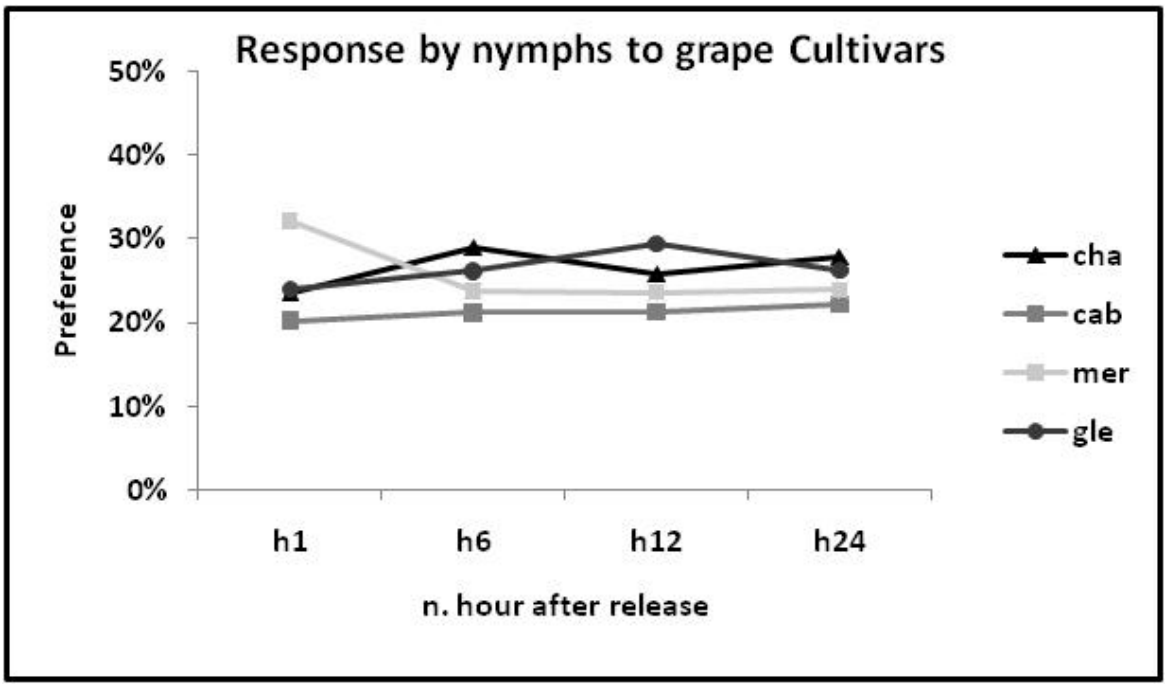

Figure 4- Preference for a Cultivar exhibited by *Scaphoideus titanus* nymphs during the experimental period. (h1: one hour after release; h6: six hours after release; h12: twelve hours after release; h24: twenty four hours after release). cha: Chardonnay; cab: Cabernet franc; mer: Merlot; gle: Glera.

A significant variation in adult proportion was found among Cultivars ($F_{3, 239}$=3.61; P=0.014). No effect of time ($F_{3, 239}$=0.10; P=0.961) nor of the interaction time*Cultivar ($F_{9, 239}$=1.18; P=0.311) were found. The proportion of adults found on Cabernet franc and Merlot were similar (t_{239}=0.75; P=0.454; figure 5). The proportion found on Cabernet franc was higher compared to that of Glera and Chardonnay (vs. Chardonnay: t_{239}=2.78; P=0.006; vs. Glera: t_{239}=2.38; P=0.014; figure 5). Merlot showed a higher adult proportion than Chardonnay, but a similar proportion compared to Glera (vs. Chardonnay: t_{239}=2.03; P=0.043; vs. Glera: t_{239}=1.73; P=0.085; figure 5). Finally, Glera and Chardonnay showed similar adult proportion (t_{239}=0.30; P=0.763; figure 5).

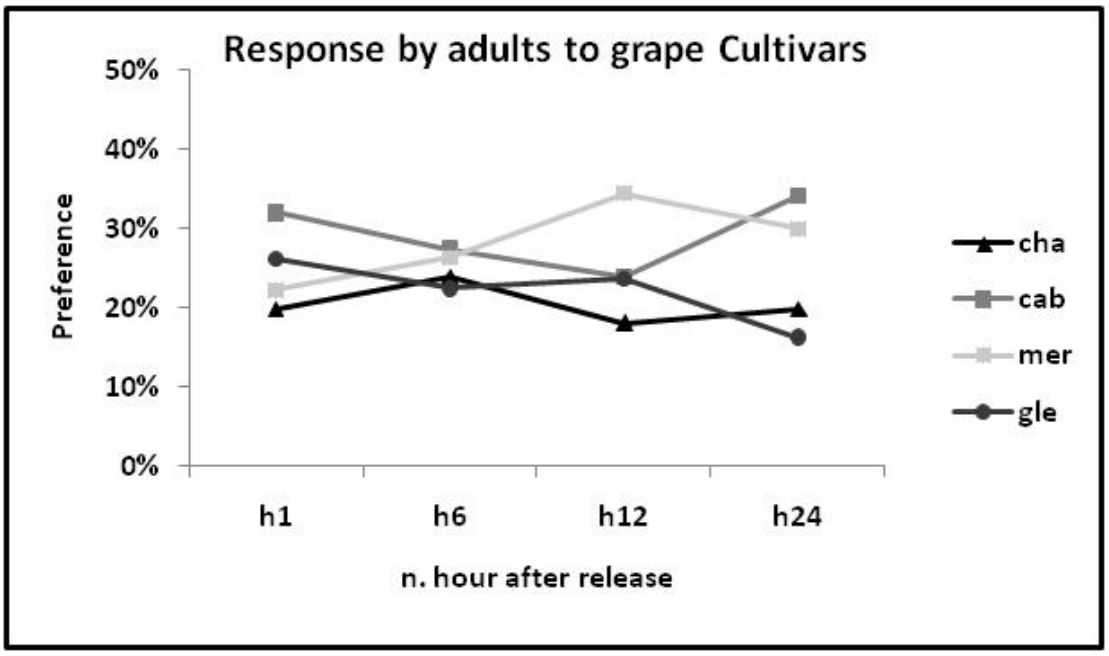

Figure 5- Preference for a Cultivar by *Scaphoideus titanus* adults during the experimental period. (h1: one hour after release; h6: six hours after release; h12: twelve hours after release; h24: twenty four hours after release). Cha: Chardonnay; cab: Cabernet franc; mer: Merlot; gle: Glera.

Scaphoideus titanus perception of stimuli emitted by healthy and infected shoots

At the beginning, *S. titanus* nymphs (n. 152) and adults (n. 75) were tested separately to study their ability to distinguish between stimuli emitted by healthy and FD infected shoots. A significant difference was found in the nymph proportion among healthy shoots, FD infected shoots and No-choice options (χ^2=87.8816, df=2, $P < 0.001$). The 69% of nymphs selected healthy shoots showing a higher attraction rate compared to FD infected shoots (18%) and No-choice option (13%) (figure 6). The proportion of nymphs which selected FD infected shoots and those which selected the No-choice option was similar. Adults also showed a different proportion among the three options (χ^2=43.44, df= 2, $P< 0.001$). The 68% of adults selected healthy shoots showing a higher attraction rate compared to FD infected shoots (24%) and No-choice option (8%) (figure 6). The proportion of adults that preferred FD infected shoots or the No-choice option was similar.

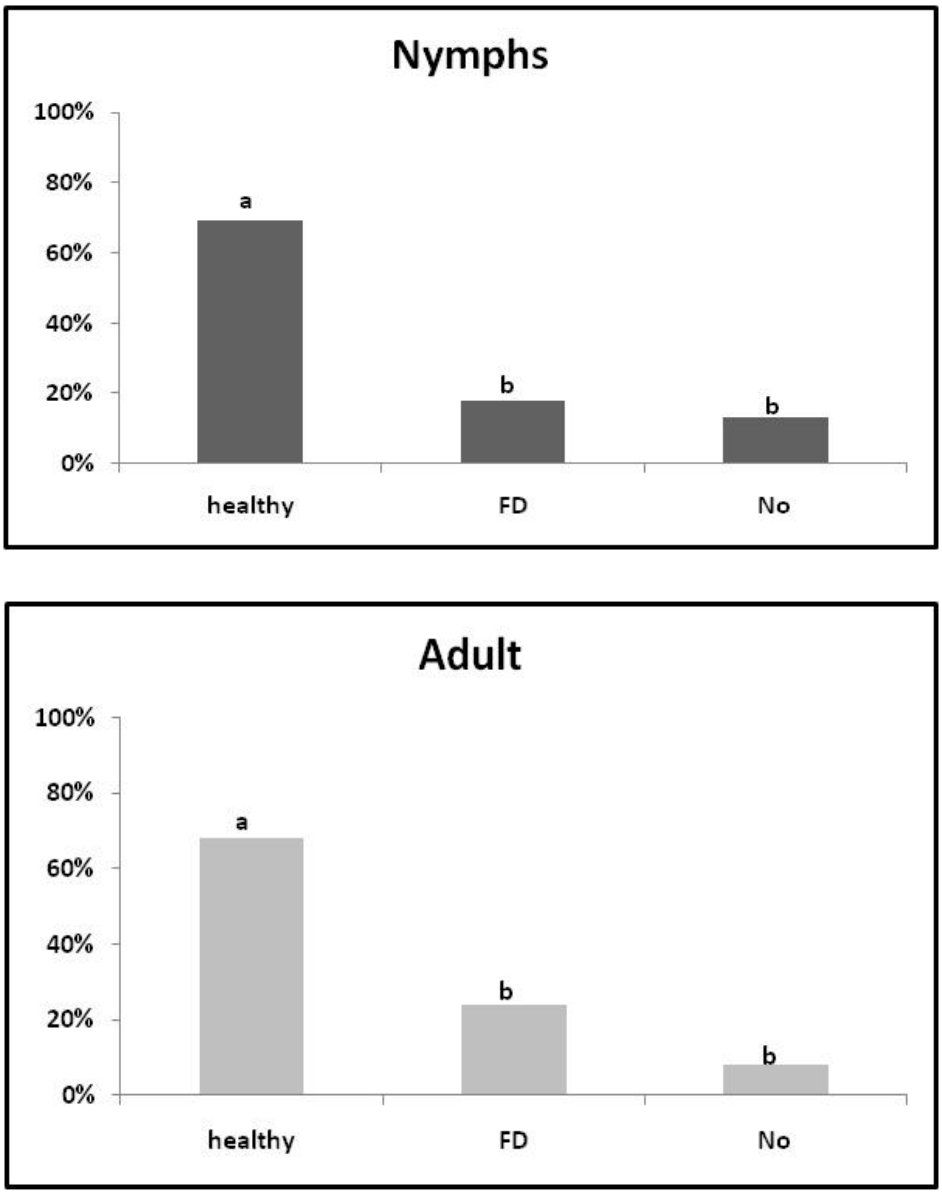

Figure 6- Comparison between healthy and FD infected shoots using the olfactometer. No (refers to No-choice), and the letters a,b indicates the significant difference according to chi-square test.

Another group of insects was tested to see if they could distinguish between the stimuli emitted by FD and BN infected shoots. No significant variation was found in the nymph proportion among the three options (χ^2=2.6, df=2, *P*=0.272). Out of 120 nymphs, 40% were attracted by FD infected shoots, 28% by BN infected shoots and 32% were for No-choice (figure 7). For adults, no significant variation in the adult proportion among the three options was found (χ^2=0.1, df=2,

P=0.951). Out of 60 adults, 32% selected FD infected shoots, 33% selected BN infected shoots and 35% did not make any decision (figure 7).

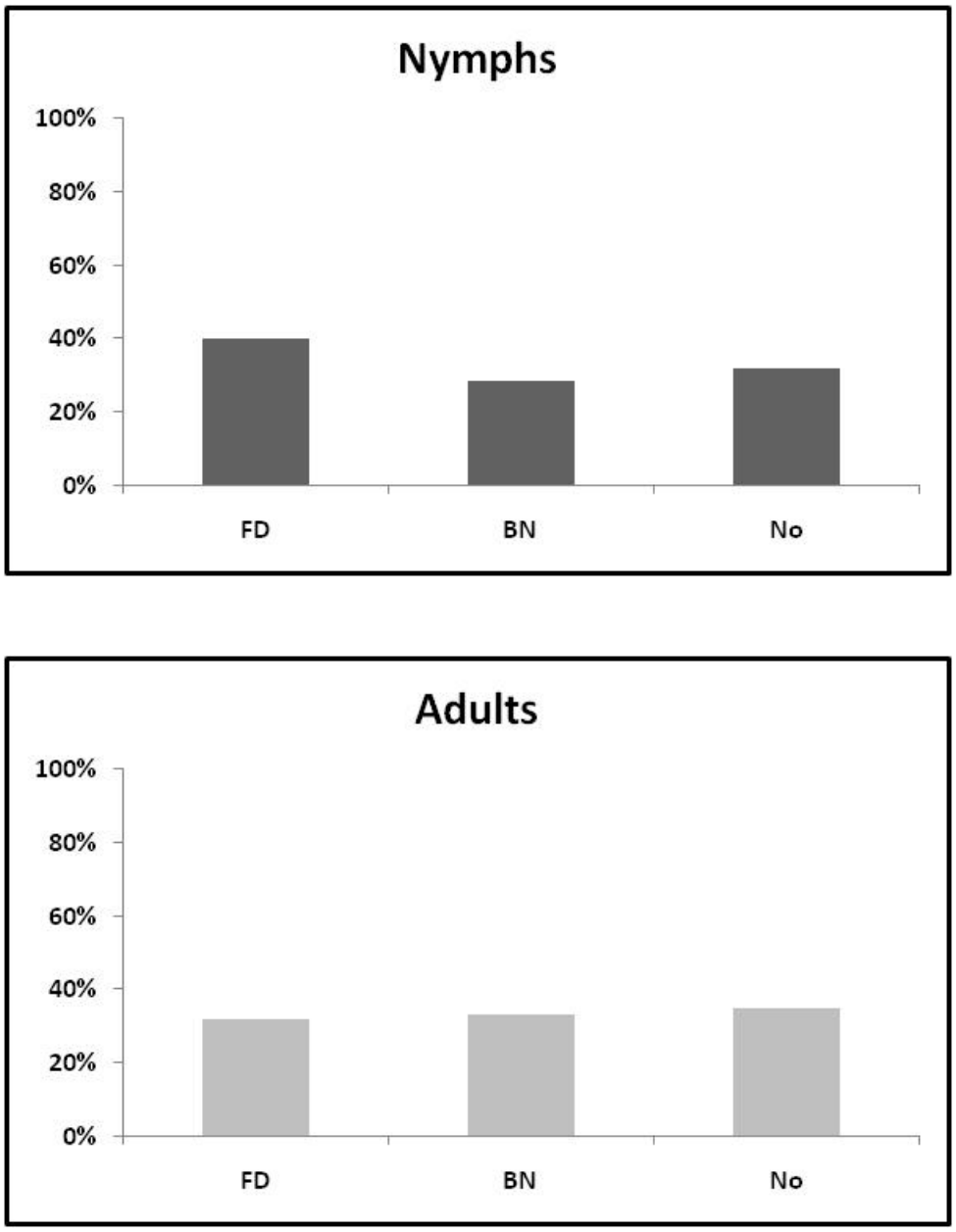

Figure 7- Comparison between FD infected and BN infected shoots using the olfactometer. No refers to No-choice,

A significant difference was found for the time needed to make the decision between the healthy and FD infected shoots due to the age of the insect (*F*= 10.863, df=1, *P*=0.0012). No effect was found due to the decision (*F*=1.348, df=1, *P*= 0.2471) nor to the effect of the interaction

age*decision (F=0.246, df=1, P=0.6206). In other words (as shown in figure 8) the insects needed less time to prefer healthy compared to FD infected shoots. At the same time, nymphs needed more time to make the decision compared to the adults.

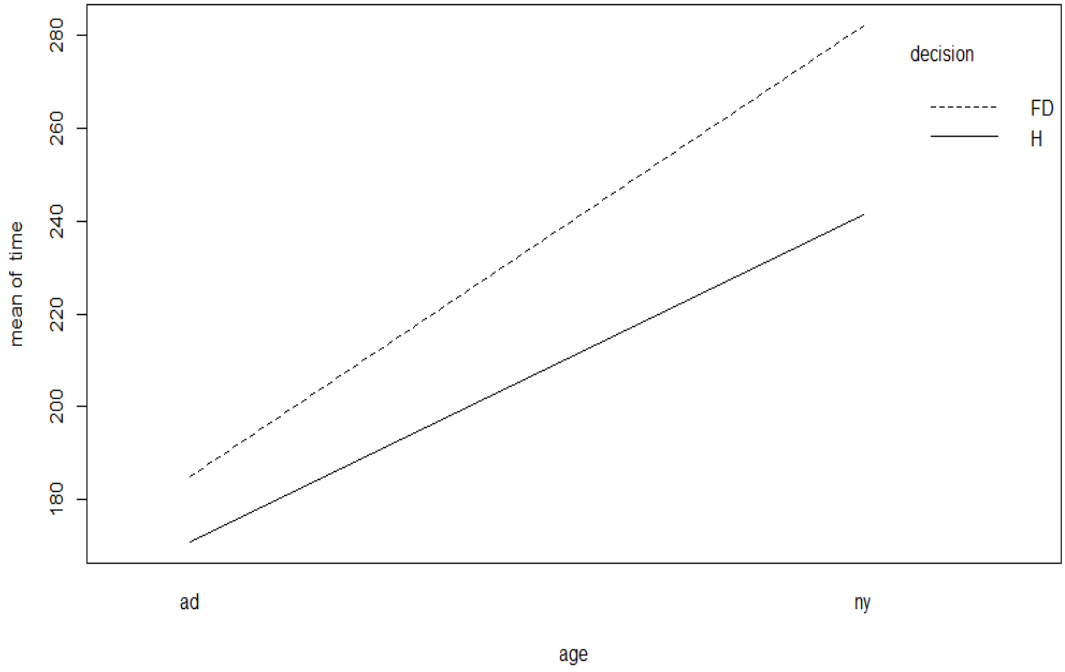

Figure 8- Interaction plot describing the decision (H: healthy and FD: FD infected shoots), and the age (ad: adults and ny: nymphs) for the time needed to make the decision (in seconds).

On the other hand, no effect was found for the age on the time needed to make the decision between FD and BN infected shoots (F=0.109, df=1, P=0.742). Decision also did not have any effect (F=2.46, df=1, P=0.12), and the interaction age*decision was not significant (F=1.932, df=1, P=0.167) (figure 9).

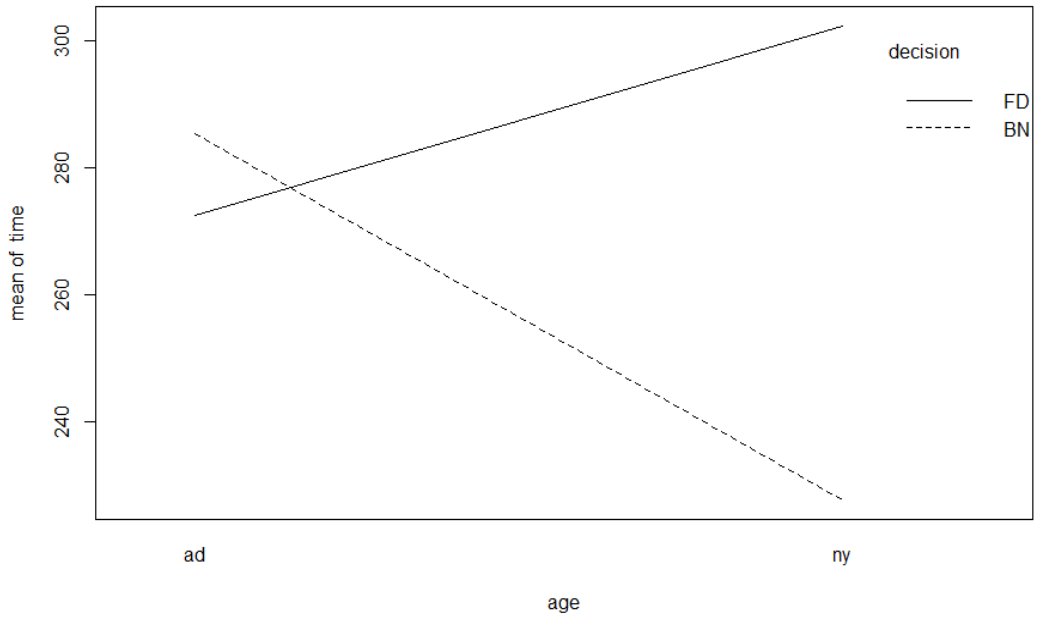

Figure 9- Interaction plot describing the decision (FD: FD infected shoots and BN: BN infected shoots), and the age (ad: adults and ny: nymphs) for the time needed to make the decision (in seconds).

Discussion

Scaphoideus titanus exhibited some preferences among Chardonnay, Cabernet franc, Merlot and Glera Cultivars because it was not attracted at the same degree by the four Cultivars. This attraction varied over the time starting from the release till one-day after (the end of the experiment). Young nymphs needed some time before showing a preference among the Cultivars, while nymphs showed no specific preferences to any of the four Cultivars after 6 hours of the release till the end of the experiment. Adults showed a preference that varied over the time of experiment.

In general, Chardonnay was more attractive for young and aged nymphs but less attractive for adults. On the other hand young nymphs and adults seemed to prefer Cabernet franc which was the least preferred Cultivar by aged nymphs. In general, all the four Cultivars were attractive for *S. titanus* but this attraction varied with time and age.

The preference of *S. titanus* for one of the Cultivars may depend on leaf characteristics (e.g., leaf hairiness). Pavan and Picotti (2009) showed that there were different susceptibility levels of

grape Cultivars toward the grape leafhopper *Empoasca vitis* (Goethe). They found that the lowest egg density was on Chardonnay and Cabernet, and that the hairiness of grape leaves did not affect the egg density but it had an effect on the parasitism rate by *Anagrus* spp.

In the case of *S. titanus*, the leaf characteristics could be the main driving force for the vector decision, because on potted plants there were no grape clusters yellow or red coloured which proved to be attractive to this insect in different proportions (Lessio and Alma, 2004). According to the hair density of different grapevine Cultivars leaves (Pavan and Peterlungher, 1999), Chardonnay has the lowest hair density compared to Merlot and Cabernet franc, except for the erect hairs on the main veins. This low hair density could be favourable for nymphs in particularly to the young ones which feed mainly from the grapevine leaf veins (Lessio and Alma, 2006). Another factor that may have influenced the insect decision is the presence of volatiles emitted by grape leaves and shoots. Olfactometer studies will be performed to test this hypothesis.

Scaphoideus titanus nymphs and adults were able to distinguish between healthy and FD infected grapevine shoots. Moreover, *S. titanus* did not exhibit preference for any of the two phytoplasma infected shoots (FD and BN), which correspond with higher tendency of *S. titanus* for the No-choice option, in this case.

Both nymphs and adults showed a similar ability to distinguish healthy shoots, so no effect of the age was found on the decision. Actually, the effect was for the time needed to make the decision and the age of the insect, as adults decided and moved faster than nymphs.

The results of this experiment showed for the first time that stimuli emitted by grapevines help *S. titanus* not only in host recognition (Mazzoni *et al.*, 2009) but also in distinguishing between healthy and FD infected grapevines shoots. This may contribute to explain the role of *S. titanus* in spreading FD within a vineyard: the attraction to healthy plants for feeding can favour the disease transmission.

Understanding the preference of *S. titanus* towards different grape Cultivars is essential for accurate pest control decisions. The most attractive Cultivars could be monitored carefully to reveal the presence of leafhoppers, thus controlling them at the right time. Some studies showed

that the spread of *S. titanus* within a vineyard is strongly related to the planting system and the canopy density (Lessio and Alma, 2004). In the establishment of new vineyards, the least preferred Cultivars could help to reduce *S. titanus* pressure and consequently FD infection. Further research is needed to know the effect of grapevine Cultivar on *S. titanus*, in particular the characteristics of each Cultivar responsible for the attractiveness towards this pest. As an example, if leaf hair density is responsible for such preference rate, then this density should be taken into consideration in hybridization programs.

On the other hand, the high attraction of *S. titanus* towards healthy shoots stresses the need to identify the responsible plant volatile compounds and probably to synthesize them. These products could be used in the traps as a tool to attract and monitor *S. titanus* for more efficient control management.

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Chapter 6

The effect of plant status on the survival and development of *Scaphoideus titanus* (Hemiptera: Cicadellidae) under controlled conditions

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Haya AbouAssaf collected part of the data, contributed to their analysis and drafted the manuscript. This work was made in cooperation with Dr. Nicola Mori, Dr. Alberto Pozzebon, Dr. Diego Fornasiero and Prof. Carlo Duso.

Abstract

Flavescence dorée (FD) and Bois noir (BN) are Grape Yellow (GY) diseases causing severe damage in European vineyards. *Candidatus* Phytoplasma vitis, the causal agent of FD disease, is transmitted from grapevine to grapevine by *Scaphoideus titanus*. *Candidatus* Phytoplasma solani, the phytoplasma associated to BN was also detected inside *S. titanus* but this cicadellid was not involved in BN transmission. The aim of this study was to understand if the interaction between phytoplasmas and *S. titanus* can result in effects on vector biology. Newly hatched nymphs of *S. titanus* were collected and reared under controlled conditions to investigate the effect of phytoplasmas on their survival and development. Healthy, FD and BN infected grapevine leaves were used for experimental treatments. The results showed that nymphs reared on phytoplasma infected leaves had longer developmental times and lower survival than those reared on healthy leaves. The interactions between *S. titanus* and the phytoplasmas could be among the factors that influence the vector population dynamics in vineyards.

Introduction

Flavescence dorée (FD) and Bois noir (BN) are Grape Yellow diseases causing severe damage in European vineyards. The disease symptoms include yellow leaves, downward curling of the leaves, fruit abortion, reduction in fruit setting, thin rubbery shoots, failure in the lignification of new shoots (Caudwell, 1983) and in some varieties the death to the plant (Boudon-Padieu, 1996; Pavan *et al*., 1997; Osler *et al*., 2002). FD affects the yield and the quality of production both for table and wine grapes (Steffek *et al*., 2007). Usually, infected plants start to express the disease symptoms after one year from the inoculation, depending on the variety and the age of plants (Caudwell *et al*., 1987). Later, a recovery could be seen unless vines are not exposed again to infection (Caudwell *et al*., 1987). In any case, the yield of the recovered plants is still lower than that of healthy ones (Morone *et al*., 2007).

Candidatus Phytoplasma vitis, the causal agent of FD disease, is transmitted from grapevine to grapevine by the leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) (Schvester *et al*., 1963; Carraro *et al*., 1994; Bianco *et al*., 2001; Mori *et al*., 2002), while BN is associated with *Candidatus* Phytoplasma solani which is transmitted to grapevines by the planthopper

Hyalesthes obsoletus Signoret (Hemiptera: Cixiidae) (Maixner 1994; Alma *et al*., 2002; Bressan *et al*., 2007). The acquisition of FD phytoplasma is performed by all the development stages of *S. titanus* but the leafhopper is able to transmit only from the third-fourth nymph instars onwards (Bressan *et al*., 2006). After a latent period the leafhopper gets involved in a vine to vine phytoplasma transmission, which causes an exponential increase of the disease infection within the vineyard (Schvester *et al*., 1969; Bressan *et al*., 2005b). *S. titanus* acquires the phytoplasma by feeding on FD infected vines but the phytoplasma is not transmitted from one generation to another by transovarial infection (Schvester *et al*., 1969; Bressan *et al*., 2006). The acquisition efficiency depends on several factors, like the susceptibility of grapevine Cultivar (Bressan *et al*., 2005a), the nymph age (Schvester *et al*., 1969) and probably the period of growing (Bressan *et al*., 2006). In spite of the fact that adults are the most efficient stage in transmitting the phytoplasma, their limited ability to spread it over long distance was proved (Lessio and Alma, 2004; 2006).

The interaction between the phytoplasma and its vector could be harmful or beneficial (Purcell, 1982). There are some studies of the effect of the phytoplasma on vectors different from *S. titanus*, like *Euscelidius variegatus* Kirschbaum, and on mechanisms of phytoplasma multiplication and invasion of different organs on this vector (Caudwell *et al*., 1972; Boudon-Padieu *et al*., 1989; Lherminier *et al*., 1990; Lefol *et al*., 1994). The effect of FD on the longevity and fecundity of *S. titanus* has been studied by confining males and females on FD infected broad beans *Vicia faba* L. (Fabaceae) (Bressan *et al*., 2005b). But most of The studies focused more on the phytoplasma acquisition by the vector than on the interaction between the vector and the phytoplasma.

The objective of this study is to shed the light on the effect of FD on the development and the survival of its natural vector *S. titanus* using grapevine leaves as phytoplasma infection source. The effect of Bois noir phytoplasma (BN) was also studied because its importance is increasing in many Italian regions (Duso *et al*., 2010) and it was detected inside *S. titanus* but this later is not involved in its transmission and spread (Carraro *et al*., 1994; Mori *et al*., 2002), the phytoplasma causal agent could have an effect on the insect biology.

Materials and methods

Plant material

Grapevine leaves of the cultivar Chardonnay were used for rearing the insects and as phytoplasma source. Healthy, FD and BN infected leaves were used. The FD infected leaves were collected from symptomatic plants in a vineyard (located in Vicenza district) where only FD was widespread. The BN infected leaves were collected from symptomatic plants in a vineyard located in Verona district where only BN was present. Healthy shoots were collected from a vineyard located in Verona district where no symptomatic plants had never been observed. The presence/absence of the phytoplasmas in the leaves was tested and certified by molecular analyses performed by CRA -VIT (Centro di Ricerca per la Viticoltura, Conegliano TV). Those leaves were provided to the insects at least twice a week.

Insects

Grapevine canes were collected from two organic vineyards located in Veneto region where the presence of *S. titanus* was ascertained; thus they were used as source of insect eggs. The canes were placed in rearing cages under screen house, and were checked daily to guarantee the accurate detection of nymphs hatching.

Artificial rearing and climatic chambers

The newly hatched nymphs (12-24 hours old) were collected and placed inside Petri dishes lined with agar and containing leaves. This method was developed by Saguez and Vincent (2011) to rear nymphs of leafhoppers belonging to the genus *Erythroneura.* Different rearing methods and relative humidity conditions were tested during 2011 in order to select the most successful for the experiment.

Then Petri dishes were placed in a climatic chamber at 24°C, relative humidity 60%, 16:8 Light: Dark ratio. This experiment was implemented in 2012 with the use of 15 Petri dishes for each group of leaves (healthy, FD and BN infected), and in each dish two nymphs were used for the study.

Molecular analyses

At the end of observations, the dead insects were tested to verify phytoplasma presence and identity. Nucleic acid was extracted according to Angelini *et al*. (2001). Nested-PCR followed by restriction fragment length polymorphism (RFLP) analyses on 16S ribosomal gene and on tuf gene for phytoplasma molecular characterization were performed as described by Duduk *et al*. (2004). Informative restriction enzymes employed were TruI and TaqI on 16S rDNA gene and HpaII on tuf gene.

Monitoring and statistical analyses

Petri dishes were monitored daily to obtain data on survival and development of *S. titanus* nymphs. The first and second nymph instars are very similar, so the presence of the exuvia was used to identify the moult. The moulting dates of other instars were easily recognized by their morphological characteristics. Data were analysed using restricted maximum likelihood model. F test ($\alpha = 0.05$) was used to evaluate the effect of treatment (FD and BN infected, healthy leaves as control) on the development times. Then, t-test on least square means was used for pairwise comparisons among the treatments. Data were transformed in logx+1 prior to the analysis to meet the model assumption. Regarding the survival, the percentage of insects which survived within each developmental stage was considered. The effect of treatments on *S. titanus* survival was evaluated with a chi-square test. Pairwise comparison of treatments was also performed with chi-square test on least square means. These analyses were performed with SAS software (SAS institute, 1999).

Results

Effects on development

A significant variation among the three treatments (healthy, FD and BN infected leaves) was observed in time needed by nymphs developing from the first to the second instar ($F_{2,22}$=4.07; P=0.031). Nymphs reared on healthy leaves have a significant shorter development time (first to second instar) than those reared on FD infected leaves (t_{22}=2.85; P=0.009; figure 1), while there were no differences between nymphs reared on BN infected leaves (t_{22}=0.81; P=0.426; figure 1).

The development time for nymphs reared on FD or BN infected leaves was also similar (t_{22}=1.75; P=0.095; figure 1).

No significant variation was observed in development times of nymphs developing from the second to the third instar ($F_{2,22}$=1.52; P=0.241). In particular, the following values were calculated: BN infected leaves vs. FD infected leaves: t_{22}=0.22; P=0.824; BN infected leaves vs. Healthy leaves: t_{22}=1.27; P=0.217; healthy leaves vs. FD infected: t_{22}=1.53; P=0.140; figure 1).

The three treatments showed significant variation in the period requested by third nymph instars to develop into fourth nymph instars ($F_{2,22}$=10.87; P<0.001). Nymphs reared on healthy leaves have a significant shorter time period than those reared on FD and BN infected leaves (vs. FD infected leaves: t_{22}=4.38; P<0.001; vs. BN infected leaves: t_{22}=2.91; P=0.008; figure 1). On the other hand, the time period for nymphs reared on BN and FD infected leaves was similar (t_{22}=1.25; P=0.223; figure 1).

No significant variation was found among the three treatments in the period requested by nymphs developing from the fourth to the fifth instar ($F_{2,22}$=0.35; P=0.707). The time period for nymphs reared on healthy, FD and BN infected leaves was similar (BN infected leaves vs. FD infected leaves: t_{22}=0.83; P=0.413; BN infected leaves vs. Healthy leaves: t_{22}=0.56; P=0.578; healthy vs. FD infected leaves: t_{22}=0.41; P=0.684; figure 1).

Also no significant variation was found among the three treatments for nymphs developing from fifth nymph instars to adults ($F_{2,22}$=0.45; P=0.644). The following values were calculated: BN vs. FD infected leaves: t_{22}=0.94; P=0.356; BN infected leaves vs. Healthy leaves: t_{22}=0.62; P=0.539; Healthy leaves vs. FD infected leaves: t_{22}=0.48; P=0.637; figure 1).

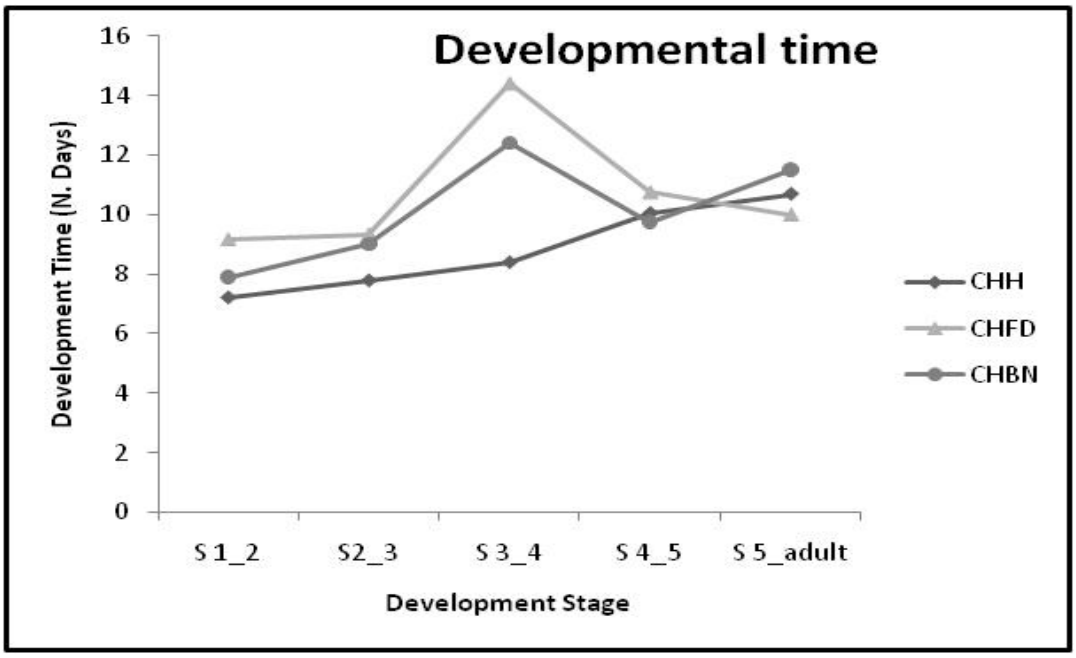

Figure 1- Developmental times (expressed in days) of different juvenile stages of *Scaphoideus titanus* reared on healthy (CHH), FD infected (CHFD) and BN infected (CHBN) leaves (S 1_2: first to second instar; S2_3: second to third instar; S3_4: third to fourth instar; S4_5: fourth to fifth instar; S5_ad: fifth instar to adult).

There was a significant effect of treatments on total development times of *S. titanus* ($F_{2,22}$=6.98; P=0.005). Insect reared on healthy leaves had the shortest times compared to those reared on FD and BN infected leaves (vs. FD: t_{22}=3.51; P=0.002; vs. BN: t_{22}=2.31; P=0.03; figure 2). Insects reared on FD and BN infected leaves showed similar time periods (t_{22}=1.02; P=0.317; figure 2).

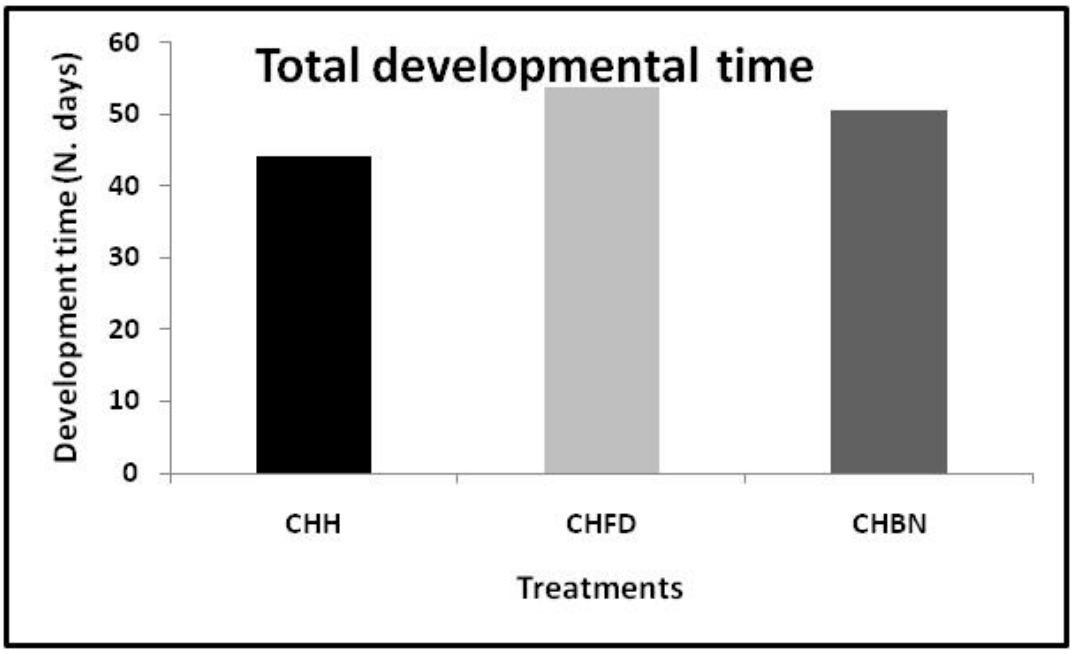

Figure 2- Total development time period of *Scaphoideus titanus* (expressed in days) reared on healthy (CHH), FD infected (CHFD) and BN infected (CHBN) leaves.

Effects on survival

A significant variation in the survival of nymphs developing from first to second instars was found (χ^2 =8.38; DF=2; *P*=0.015). The survival of nymphs reared on healthy and BN infected leaves was similar (χ^2 =0.00; DF=1; *P*=1.000; figure 3), while the survival of insects reared on FD infected leaves was lower than that of those reared on healthy or BN infected leaves (vs. Healthy leaves: χ^2 =3.88; DF=1; *P*=0.049; vs. BN infected leaves: χ^2 =3.88; DF=1; *P*=0.049; figure 3).

Nymphs developing from second to third instars showed a significant variation in the survival among the three treatments (χ^2 =16.07; DF=2; *P*<0.001). The survival of nymphs reared on healthy and BN infected leaves was similar (χ^2 =0.00; DF=1; *P*=1.000; figure 3), but those reared on FD infected leaves survived less than those reared on healthy (χ^2 =6.49; DF=1; *P*=0.011) or BN infected leaves (χ^2 =6.49; DF=1; *P*=0.011; figure 3).

A significant variance in the survival among the three treatments was found for nymphs developing from third to fourth instars (χ^2 =8.87; DF=2; *P*=0.012). The survival of nymphs reared on healthy and BN infected leaves was similar (χ^2 =0.57; DF=1; *P*=0.452; figure 3). The

lowest survival value was found for nymphs reared on FD infected leaves (vs. Healthy leaves: χ^2 =4.25; DF=1; *P*=0.039; vs. BN infected leaves: χ^2 =6.75; DF=1; *P*=0.009; figure 3).

No significant variance was found among the three treatments in the survival of nymphs developing from fourth to fifth instars (χ^2 =4.29; DF=2; *P*=0.1173). The following values were calculated: healthy leaves vs. BN infected leaves: χ^2 =3.73; DF=1; *P*=0.054; healthy leaves vs. FD leaves: χ^2 =2.25; DF=1; *P*=0.134; FD infected leaves vs. BN infected leaves: χ^2 =0.10; DF=1; P=0.746; figure 3).

A significant variation was found in the survival among the three treatments for nymphs developing from fifth instar to adults (χ^2 =7.98; DF=2; *P*=0.018). Nymphs reared on healthy leaves have the highest survival value compared to the other two treatments (vs. BN infected leaves: χ^2 =5.91; DF=1; *P*=0.016; vs. FD infected leaves: χ^2 =4.87; DF=1; *P*=0.027; figure 3). On the other hand, nymphs reared on FD and BN infected leaves have similar survival values (χ^2 =0.01; DF=1; P=0.908; figure 3).

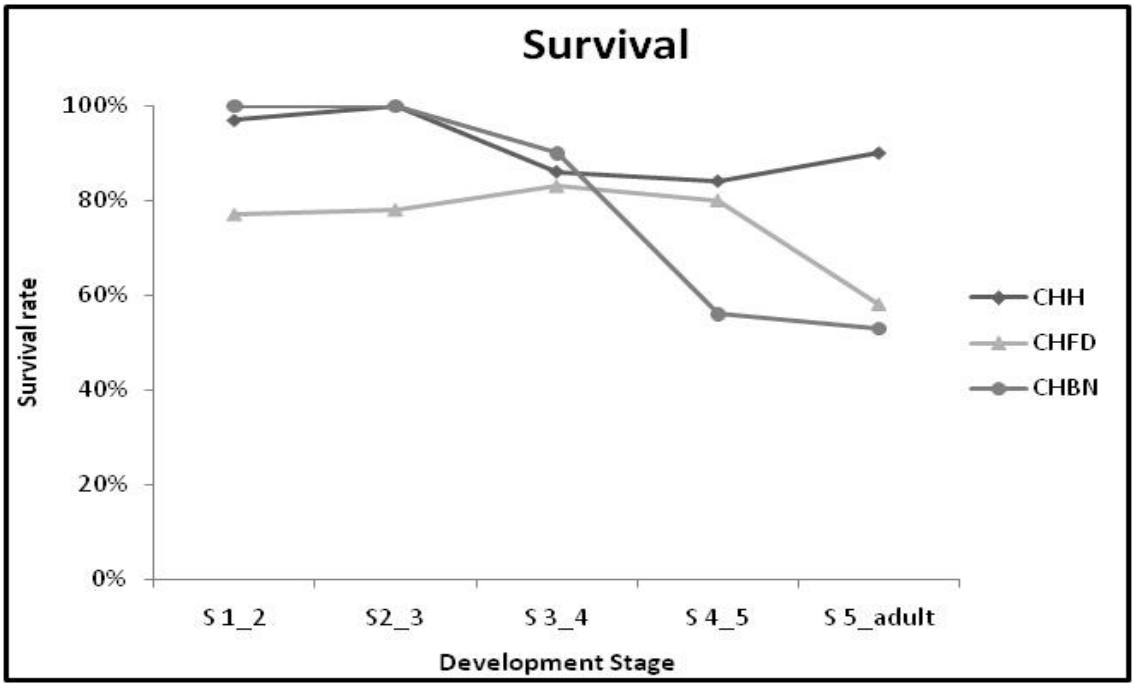

Figure 3- The survival of the various development stages of *Scaphoideus titanus* reared on healthy (CHH), FD infected (CHFD) and BN infected (CHBN) leaves (S 1_2: first to second instar; S2_3: second to third instar; S3_4: third to fourth instar; S4_5: fourth to fifth instar; S5_ad: fifth instar to adult).

The effect of treatments on the survival of *S. titanus* nymphs was significant (χ^2 =12.51; DF=2; *P*=0.002). Nymphs reared on healthy leaves had the highest survival compared to those reared on FD and BN infected leaves (vs. FD infected leaves: χ^2 =9.14; DF=1; *P*=0.003; vs. BN infected leaves: χ^2 =7.73; DF=1; *P*=0.005; figure 4). No difference was found in the survival of nymphs reared on FD and BN infected leaves (χ^2 =0.09; DF=1; *P*=0.766; figure 4).

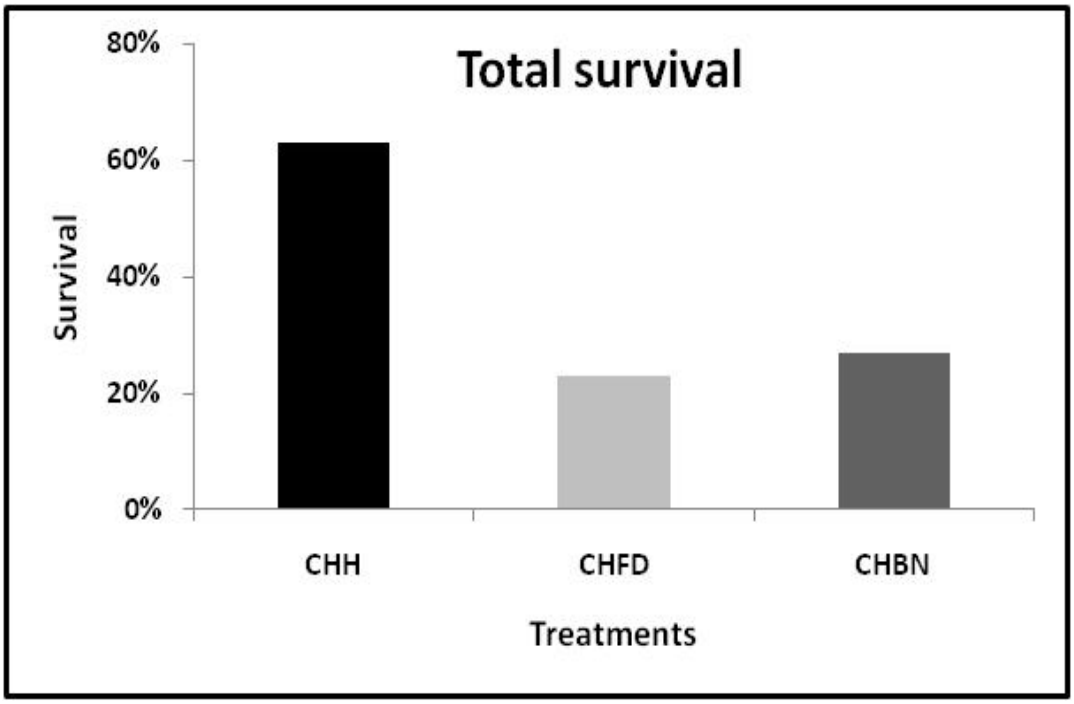

Figure 4- Survival of nymphs on different treatments.

Result of molecular analyses

The results of molecular analyses showed the presence of the phytoplasma 16SrV, the causal agent of FD, in 67% of leafhoppers reared on FD infected leaves. Most of them (about 20) were adults. The BN causal agent 16SrXII was detected in the 20% of insects reared on BN infected leaves. No phytoplasmas were detected on insects reared on healthy leaves.

Discussion

The results of this study show that feeding on phytoplasma infected leaves increases the developmental times of *S. titanus* and reduces its survival. Further investigations are needed to

investigate whether this effect resulted from the multiplication and the invasion of the phytoplasma inside the leafhopper body or from biochemical changes induced by the phytoplasma on grape leaves.

The effect of phytoplasmas (FD and BN) on the development and the survival of its vector could be among the factors that affect *S. titanus* populations dynamics in vineyards. A longer development time period for nymphs might expose them longer to unsuitable climatic conditions and natural antagonists. Moreover, the lower survival of nymphs will imply a reduction in the number of adults, the most effective life stages in transmitting the phytoplasma disease to grapevine, and subsequent FD infection.

Results reported in other studies can contribute to understand better this phenomenon. Bressan *et al*. (2005b) found that the experimental infection with FD phytoplasma reduced greatly the fecundity and the survival of *S. titanus* adults. The source of phytoplasma infection in Bressan *et al*. (2005b) was from broad beans, while in the present work the source of infection was grapevine, the natural host of *S. titanus*. A study on *Colladonus montanus* Van Duzee, the vector of Western X-disease phytoplasma also found that phytoplasma reduced the survival of its vector (Jensen *et al*., 1967).

In other studies phytoplasmas increased the developmental times of their vectors (Beanland *et al*., 2000; Ebbert and Nault, 2001; Kaul *et al*., 2009; Johannesem *et al*., 2011). Nevertheless, the Eastern-X disease phytoplasma was found to reduce the life span of its vector *Paraphlesius irroratus* Say (Garcia-Salazar *et al*., 1991).

The results of the present work and the previously mentioned papers reveal a various range of interactions between the phytoplasma and its vector. It has been suggested that the long evolution between them could help achieving a beneficial relation (Purcell, 1982). The negative impact of FD and BN on *S. titanus* could be due to the limited co-evolution time period (Bressan *et al*., 2005b).

The phytoplasma detection analyses confirmed the ability of *S. titanus* to acquire the BN phytoplasma but there is no proof of any involvement of *S. titanus* in the transmission of this phytoplasma.

This present work represents a preliminary step on understanding the impact caused by phytoplasma FD and BN on *S. titanus* using its natural host i.e. grapevine. This interaction could be used to develop a model for the phytoplasma epidemiology to improve *S. titanus* and FD management. In fact, a similar model has been pointed out for aster yellows on lettuce (Beanland *et al*., 2000 reported unpublished data by C.W.H.).

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Conclusions

The phenological studies of grape berry moths (*Lobesia botrana* and *Eupoecilia ambiguella*) and *Scaphoideus titanus* shed the light on the occurrence of these pests in several sites in Veneto region. According to the results of this survey, the presence of grape berry moths was not homogeneous in the studied areas. There was a clear dominance of *E. ambiguella* in hilly areas and of *L. botrana* in the plain areas confirming previous reports. In one of these sites (Valdobbiadene) both species were detected but in a fluctuating rate through the studying period. Temperature could be a major factor explaining this fluctuation as the relation between temperature and second flight was found to be significant for *E. ambiguella* and *L. botrana*. Two to three generations were seen for *E. ambiguella*, while *Lobesia botrana* achieved three generations in most of the sites. The third flight was sometimes unusually long which may suggest a larval aestivation. Four adult peaks were observed for *L. botrana* in some areas, but no evidence for a fourth generation was provided.

Regarding the phenology of *S. titanus*, nymphs appeared in the second half of May and the adults from the end of June to early July. This corresponds with previous studies on the phenology of *S. titanus* in Veneto region. However, the results of this work showed a significant variation in the phenology of *S. titanus* among the sites and the years. This variation could not by explained only by temperature, as other environmental factors such as altitude and sun exposure are probably involved. Nevertheless, a significant effect of temperature on the appearance of nymphs was found suggesting its major role on *S. titanus* phenology. In contrast, the relation between temperature and nymph development was not clear suggesting the need for further studies.

In most sites the phenology of grape berry moths (second flight) and *S. titanus* (first appearance of nymphs/adults) was earlier in 2011 compared to 2010 and 2012 and this effect was likely associated to spring temperatures of 2011. Phenological data are essential for developing forecasting models to help in predicting the occurrence of those pests in a given area and improve their control according to current EC rules (e.g., Directive 128/2009).

The population diversity and the genetic structure of *L. botrana* were investigated. Microsatellite loci were used to analyse 16 *L. botrana* populations from Europe (Spain, Italy and Germany) and

the Middle East (Syria, and Golan heights). A moderate level of genetic differentiation was found among the populations. However, the low flight ability of this moth could explain this. The highest genetic differentiation level was found with an Italian population from Meolo (Veneto region, Italy). But, the high heterozygosity values for most of the studied populations suggest that a recent breaking in isolation took place enabling the individuals to mate among the different populations. The AMOVA results confirmed also the high heterozygosity within the individual level with no clear effect of the geographic location. This later result corresponded with what was found with the STRUCTURE analyses. The European populations (Italian and German) are not strongly structured and that they appear to be a mixed of two genotypes, the first genotype was noticed with the Spanish population and the second with the populations of Middle East. The evaluation of gene flow among the populations and the level of diversity of their individuals is essential to understand the origin, the distribution and the potential expansion of an insect pest to new areas.

The behaviour of *S. titanus* and the factors which affect its distribution within vineyards were investigated in a number of laboratory and semi-field experiments.

Grapevine cultivars may have a role in the pest distribution in viticultural areas. Therefore, the response by *S. titanus* to four grapevine varieties (Chardonnay, Cabernet franc, Merlot and Glera) was tested in semi-field conditions. The leafhopper was able to distinguish among the selected varieties and exhibited some preferences. Chardonnay seemed to be preferred by the young nymphs (first and second instars) and aged nymphs (third, fourth and fifth instars) but was less preferred by adults. Cabernet franc, on the other hand, was more preferred by young nymphs and adults compared to aged nymphs. Leaf characteristics could be a reason for this variation in the preference by nymphs and adults.

The ability of *S. titanus* to distinguish among healthy and infected stimuli was investigated using an olfactometer. Both nymphs and adults were able to distinguish between healthy and Grape Yellow phytoplasma (Flavescence dorée and Bois noir) infected shoots, and they were significantly attracted by stimuli emitted from healthy shoots. Leafhoppers moved faster towards healthy shoots while nymphs seemed to need more time to make a decision. *S. titanus* was not able to distinguish between the stimuli emitted by Flavescence dorée (FD) or Bois noir (BN) infected shoots. These results could help in understanding the spread of *S. titanus* within

vineyards. The stimuli emitted by shoots help in host recognition and in detecting the plant status (healthy or GY infected).

Finally, the effect of phytoplasmas on *S. titanus* biology was studied by rearing leafhopper nymphs on healthy or GY (FD or BN) infected leaves. Grapevine leaves infected by FD and BN increased the developmental times of *S. titanus* nymphs and decreased their survival when compared to healthy leaves. The results of this study contribute to the knowledge of interactions between phytoplasmas, vectors and host plants. However, mechanisms involved in these effects require additional investigations.

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