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## **THE IMPACT OF PESTICIDES ON APPLE MITE COMMUNITIES**

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December, 31st 2012

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## Riassunto

Nell'ambito di questo lavoro sono stati condotti esperimenti di campo, semi-campo e laboratorio sugli acari predatori *Kampimodromus aberrans*, *Amblyseius andersoni*, *Typhlodromus pyri* e *Phytoseius finitimus* allo scopo di perfezionare le strategie di controllo biologico degli acari fitofagi nei meleti. Il primo esperimento ha riguardato l'introduzione di *K. aberrans* in meleti convenzionali e biologici. I meleti biologici erano trattati soprattutto con piretro e spinosad, mentre i meleti convenzionali erano trattati con neonicotinoidi, esteri fosforici e regolatori della crescita. Le densità dei predatori hanno raggiunto densità superiori nelle tesi oggetto d'introduzione rispetto ai testimoni. Tuttavia, le popolazioni dei predatori hanno raggiunto livelli più elevati nei meleti biologici rispetto ai meleti convenzionali. *Amblyseius andersoni* risultava naturalmente presente nei meleti. Nel 2010, non sono stati riscontrati effetti delle introduzioni di *K. aberrans* né della gestione dei meleti sull'abbondanza di *A. andersoni*. Al contrario, nel 2011 sono state riscontrate densità più contenute di *A. andersoni* nelle tesi oggetto d'introduzione rispetto ai testimoni. In uno dei siti sperimentali (Spresiano), le popolazioni di *K. aberrans* sono risultate più elevate, ove introdotte, in due meleti di cultivar Florina e Golden Delicious. Le densità di *K. aberrans* sono aumentate nel corso del tempo ma in misura più rilevante su Florina, probabilmente per la morfologia fogliare di questa cultivar. Nel meleto di Golden Delicious, le popolazioni di *A. andersoni* sono risultate più contenute nella tesi oggetto d'introduzione di *K. aberrans* rispetto ai testimoni; probabilmente, questo risultato è stato influenzato dalla morfologia fogliare di questa cultivar. Nei meleti convenzionali, le introduzioni di *K. aberrans* sono fallite a causa di trattamenti ripetuti con prodotti fitosanitari non selettivi ma anche gli insetticidi impiegati nei meleti biologici hanno comportato decrementi significativi delle densità dei predatori.

La competizione tra *K. aberrans*, *A. andersoni*, *T. pyri* e *P. finitimus* è stata oggetto di un secondo esperimento. Le femmine dei predatori sono state allevate sulle larve delle altre specie allo scopo di simulare possibili interazioni in condizioni di scarsità di preda. In queste condizioni sperimentali, i predatori hanno esibito buoni tassi di sopravvivenza e di fecondità. *A. andersoni* ha predato più larve di *T. pyri* e *P. finitimus* che di *K. aberrans* e la sua fecondità è stata superiore sulle prime due prede. Per *A. andersoni*, una dieta basata sulle larve di *T. pyri* è stata associata a tassi più elevati di conversione delle prede in biomassa destinata alle uova; la longevità del predatore è risultata più elevata sulle larve di *K. aberrans*. *K. aberrans* ha predato più *P. finitimus* che *T. pyri* e *A. andersoni* e la sua fecondità è risultata più elevata sulla prima specie. Il tasso di conversione in uova non ha mostrato tendenze particolari mentre la longevità di *K. aberrans* è risultata più elevata quando i predatori sono stati alimentati con

larve di *A. andersoni*. *Typhlodromus pyri* ha consumato più larve di *P. finitimus* che di *A. andersoni* e il tasso di ovideposizione è stato conseguente. *Phytoseius finitimus* ha predato più larve di *K. aberrans* e *T. pyri* che di *A. andersoni* ma la fecondità non ha mostrato la stessa tendenza: i predatori allevati su *A. andersoni* sono stati più efficienti nella conversione delle prede in uova. Relativamente ai tassi di predazione e di fecondità, *A. andersoni* sembra avvantaggiato rispetto a *T. pyri*, *T. pyri* rispetto a *K. aberrans*, mentre *A. andersoni*, *K. aberrans* e *T. pyri* sono avvantaggiati rispetto a *P. finitimus*. Le prestazioni di *A. andersoni*, *K. aberrans* e *T. pyri* (tassi di predazione e fecondità) sono state migliori quando i predatori sono stati allevati con larve di *P. finitimus*. Le prestazioni di *P. finitimus* sono apparse le più basse in assoluto e questo suggerisce uno svantaggio competitivo. L'alimentazione pollinica ha garantito prestazioni ottimali in termine di fecondità a sostegno del ruolo del polline nel garantire la persistenza dei predatori generalisti negli ecosistemi agrari.

Il terzo esperimento ha riguardato l'effetto degli insetticidi su *K. aberrans* in condizioni di pieno campo e di laboratorio. E' stato saggiato l'effetto degli insetticidi chlorpyrifos, thiacloprid, acetamiprid, lufenuron, indoxacarb, methoxyfenozide e etofenprox. Nelle prove di pieno campo, etofenprox è risultato l'insetticida più dannoso nei confronti degli acari predatori ed ha causato pullulazioni di acari fitofagi. In ulteriori prove di pieno campo, spinosad e tau-fluvalinate hanno causato problemi analoghi. Nelle prove di laboratorio sono stati inseriti anche spinosad, tau-fluvalinate, thiamethoxam, clothianidin e imidacloprid. Spinosad, tau-fluvalinate e etofenprox hanno causato effetti drammatici sulla sopravvivenza e sulla fecondità di *K. aberrans*. I neonicotinoidi non sono apparsi molto tossici ma hanno causato una riduzione della fecondità di *K. aberrans*. Per altri insetticidi (chlorpyrifos, indoxacarb, lufenuron e methoxyfenozide) sono state osservate riduzioni meno drammatiche della fecondità del predatore.

In un altro studio è stato valutato il ruolo del polline nel mitigare gli effetti degli insetticidi su *K. aberrans*. Nella prima parte di questo studio sono stati calcolati i parametri demografici di *K. aberrans* allevato su polline o su *Panonychus ulmi*. Il polline si è dimostrato un alimento ottimale rispetto a *P. ulmi* in quanto i predatori hanno esibito una fecondità più elevata e tempi di sviluppo più brevi con implicazioni per i parametri demografici. La seconda parte dell'esperimento ha riguardato le interazioni tra pesticidi, polline e *K. aberrans* in condizioni di semi-campo. Gli acari predatori sono stati introdotti 7 giorni prima dei trattamenti insetticidi e il polline di *Typha latifolia* è stato impiegato quale alimento. L'applicazione degli insetticidi (soprattutto spinosad) ha ridotto la densità della popolazione di *K. aberrans*. L'applicazione ripetuta del polline è stata associata a un incremento della popolazione dei

predatori. E' stata riscontrata un'interazione significativa tra l'applicazione di polline e degli insetticidi: l'applicazione di polline ha mitigato l'effetto del chlorpyrifos sulla fecondità di *K. aberrans*. Tuttavia, questo effetto non è stato osservato sulle piante trattate con spinosad.

I meccanismi di resistenza al chlorpyrifos in *K. aberrans* sono stati oggetto di un ultimo contributo. In particolare, è stata indagata la resistenza associata a mutazioni nell'enzima Acetilcolinesterasi (AChE). Il cDNA dell'enzima è stato clonato mediante una strategia basata sul sequenziamento della AChE di *Tetranychus urticae*. Al fine di ottenere sequenze omologhe di cDNA in *K. aberrans* sono stati disegnati primers con domini funzionali delle AChE nel genoma dell'acaro predatore *Metaseiulus occidentalis*. Combinando le sequenze AChE di ceppi sensibili e resistenti di *K. aberrans* è stata posta in evidenza una sostituzione di glicina in serina (G119S) mentre entrambe le sequenze AChE avevano un residuo di fenilalanina in posizione 331. Questi risultati hanno escluso la mutazione F331W quale responsabile della resistenza target size al chlorpyrifos ma apre interrogativi sul ruolo della mutazione G119S. Questa sostituzione è già stata descritta in *Anopheles gambiae* e *Culex pipiens* ove è coinvolta in una AChE1 insensibile o a ridotta attività. Tale sostituzione è stata associata ad una moderata resistenza al chlorpyrifos in *T. urticae*. Pertanto, sembra che tale sostituzione abbia un ruolo specie-specifico. Le analisi hanno dimostrato la presenza della sostituzione G119S in tre ceppi resistenti di *K. aberrans* in cui è assente la mutazione F331W. Allo stesso tempo, due ulteriori ceppi sensibili a chlorpyrifos hanno esibito una glicina in posizione 119, in luogo della serina, nella sequenza AChE. I dati suggeriscono che il polimorfismo G119S sia coinvolto nel controllo poligenico del fenotipo resistente. Le mutazioni della AChE potrebbero essere collegate con uno o più fattori genetici determinanti la resistenza a chlorpyrifos in *K. aberrans*.



## Summary

Laboratory, field and semi-field experiments have been carried out on the predatory mites *Kampimodromus aberrans*, *Amblyseius andersoni*, *Typhlodromus pyri* and *Phytoseius finitimus* in order to optimize biological control strategies against phytophagous mites in apple orchards. The first experiment concerned the augmentative releases of *K. aberrans* in organic and conventional apple orchards. The most frequent insecticides used in organic orchards were pyrethrins and spinosad, whereas neonicotinoids, OPs and IGRs were used in conventional orchards. *K. aberrans* releases were successful and predatory mites were significantly higher in released than in control plots. Moreover, populations were high in organic orchards compared to conventional orchards. *Amblyseius andersoni* was naturally occurring in all orchards. In 2010, no effect of *K. aberrans* release and orchard management were observed on *A. andersoni* populations but in 2011 *A. andersoni* population densities were low in released plots compared to control plots. In organic orchards located at Spresiano releases were made on two cultivars, i.e., Florina and Golden Delicious. In both 2010 and 2011 releases, *K. aberrans* population densities were higher in release than in control plots of both cultivars. *K. aberrans* population increased during the three years on Florina but not in Golden Delicious orchard. On the latter *A. andersoni* population densities were lower in released than in control plots. In conventional orchards *K. aberrans* did not establish probably because of a series of non-selective insecticide and fungicide treatments. However, the use of spinosad was associated to a decline of predatory mite populations in organic orchards.

The second experiment considered interspecific predation among *K. aberrans*, *A. andersoni*, *T. pyri* and *P. finitimus*. All four predators fed with heterospecific larvae were able to survive some weeks laying a number of eggs. *A. andersoni* consumed more *T. pyri* and *P. finitimus* than *K. aberrans* larvae and its fecundity was higher on the first two prey species. A diet based on *T. pyri* larvae was associated to the highest conversion rate of food into egg biomass; longevity was higher for predatory mite females fed with *K. aberrans* larvae. *K. aberrans* preyed more *P. finitimus* than *T. pyri* and *A. andersoni* larvae and its fecundity resulted higher on the first species. The conversion of food into egg biomass did not show precise trend while the longevity of *K. aberrans* resulted higher when fed with *A. andersoni*. *Typhlodromus pyri* consumed more *P. finitimus* and *K. aberrans* than *A. andersoni* larvae and laid more eggs on the first two species. *Phytoseius finitimus* preyed more *K. aberrans* and *T. pyri* than *A. andersoni* larvae but fecundity was not affected by prey; predatory mite females fed with *A. andersoni* larvae were more efficient in converting food into egg biomass but those fed with *K. aberrans* larvae lived longer. In terms of predation rate and fecundity, *A.*

*andersoni* seems to be advantaged over *T. pyri*, *T. pyri* over *K. aberrans*, and *A. andersoni*, *K. aberrans* and *T. pyri* over *P. finitimus*. The performance of *A. andersoni*, *K. aberrans* and *T. pyri* in terms of predation rate and fecundity proved to be better of *P. finitimus* larvae than on other prey. The low prey consumption and fecundity of the latter suggest that is disadvantaged in interspecific predation. The comparison between pollen and prey diets confirmed the positive effect of pollen on the fecundity of all four predatory mite species.

The third experiment deals with the effects of insecticides, frequently applied in apple orchards, on *K. aberrans* in field and laboratory conditions. Insecticides including chlorpyrifos, thiacloprid, acetamiprid, lufenuron, indoxacarb, methoxyfenozide and etofenprox were tested. In field studies etofenprox was the most detrimental pesticide to predatory mites. It caused dramatic effects on *K. aberrans* populations and induced spider mite outbreaks. In other trials tau-fluvalinate and spinosad did similar effects. In laboratory studies we assessed lethal and sub-lethal effects for each pesticide tested. Spinosad, tau-fluvalinate, thiamethoxam, clothianidin and imidacloprid were included. Etofenprox, spinosad with tau-fluvalinate proved to be harmful insecticides to *K. aberrans* in terms of lethal effects. Neonicotinoids did not affect survival so much but their sub-lethal effects were more prominent in terms of fecundity. Other pesticides such as chlorpyrifos, indoxacarb, lufenuron and methoxyfenozide also significantly reduced fecundity in *K. aberrans* at a lower level.

In the fourth study we explored the role of pollen in alleviating the effects of pesticides on *K. aberrans* at individual and population levels. In the first part we tested biological and demographic parameters of *K. aberrans* on pollen and *Panonychus ulmi*. *K. aberrans* was reared individually on *P. ulmi* and on pollen. Both food sources allowed for predatory mites survival, development and reproduction. Pollen was preferred food as compared to *P. ulmi*. Total fecundity and survival rates of *K. aberrans* were higher on pollen than on tetranychids. Developmental times of protonymphs, deutonymphs and adults of *K. aberrans* were longer on *P. ulmi* than on pollen. Life-table parameters of *K. aberrans* were positively affected by pollen. The second part deals with the interactions of pesticides, pollen and *K. aberrans* in semi-field conditions. Experimental factors were: insecticide application and pollen application. Predatory mites were released on 3-4 shoots per plant 7 days before insecticide applications. *Typha latifolia* pollen was used. Insecticide application, particularly spinosad, determined a reduction in *K. aberrans* abundance. Pollen applications were associated to an increase in predatory mite population. Significant interaction between pollen applications was observed: pollen application mitigated the effect of chlorpyrifos (because of its compensation of sublethal effects on fecundity) but no effect was observed on spinosad treated plants.

In the last work mechanisms of resistance to chlorpyrifos due to mutations in insensitive Acetylcholinesterase (AChE) in *K. aberrans* was investigated. AChE cDNA was cloned. The cloning strategy relied on the AChE sequence of *Tetranychus urticae*. To obtain the homologous cDNA sequence in *K. aberrans*, degenerate primers were designed on conserved functional domains of the annotated AChEs in *Metaseiulus occidentalis* genome, and combined with 5'-3' rapid amplification of cDNA ends. When the cloned AChE sequence was compared between *K. aberrans* sensible and resistant strains, only a Glycine to Serine (G119S) substitution was detected while both AChE sequences had a Phenylalanine residue at 331 position. These results excluded the F331W mutation as responsible for chlorpyrifos target site resistance in *K. aberrans* but it opened interrogatives about the role of G119S. Indeed the G119S substitution had been already described in mosquitoes *Anopheles gambiae* and *Culex pipiens* where it led to AChE1 insensitivity or reduced AChE1 activity. On the contrary, the same aminoacid change in *T. urticae* AChE sequence was associated with a more moderate decrease in chlorpyrifos response. So a species-specific effect of this substitution cannot be kept out. In any case, site oriented sequencing of AChE cDNA, confirmed the presence of G119S substitution in further three resistant strains of the mite predator as well as the absence of F331W mutation. At the same time, two additional sensible strains, displayed a Glycine at the position 119, instead of Serine, in their AChE sequence. Altogether, these findings might suggest that the G119S polymorphism was involved in the polygenic control of the resistant phenotype. In addition or alternatively, the mutated AChE might be in linkage with one of the genetic determinants which affect the insecticide sensitivity in *K. aberrans*.

# **Chapter I**

## **Introduction**



## Mites

Mites are microscopic and tiny creatures belonging to subclass Acari of the class Arachnida. They are a diverse group which is worldwide in distribution and inhabiting all types of terrestrial (plants, mountains, deserts, plains, pastures) and aquatic habitats (oceans, rivers, springs, streams, lakes) (Evans, 1963 Krantz, 1959 Chillar *et al.*, 2007). Mites have always attracted considerable interest because of remarkable habits of some species. Mites may be classified as phytophagous, predatory, parasitic and stored product mites on the basis of their feeding behavior.

Tetranychid mites, the popularly called spider mites are obligate plant feeders and several species are reported to show secondary pest outbreaks (Helle and Sabelis, 1985a). There are dozens of species of spider mites belonging to genera *Tetranychus*, *Eutetranychus*, *Eotetranychus*, *Schizotetranychus*, *Oligonychus*, *Panonychus* and so on, which cause severe damage to plants. The present day knowledge on spider mites undoubtedly confirmed them as major agricultural pests, in fact they have been considered as a constant source of threat to the economy of agriculture. Tetranychid mites have been reported as pests of more than 150 cultivated plants ranging from greenhouse to fruit and tree crops. This group of mite attacks cotton, peanut, bean, eggplant, squash, cucumber, corn, apple, peach, citrus, grapevine, papaya, castor, mulberry, rose and many other ornamental plants (Naher and Haque, 2007). Some mites transmit viruses e.g. wheat streak mosaic virus by eriophyid mites (Hong *et al.*, 1999) and potato virus by tetranychid mites (Jeppson *et al.*, 1975). Chemical control of these mites is quite expensive and quite often useless as in several instances these mites are seen to develop resistance to various kinds of acaricides. Hence, there is an increasing trend to devise control measures against mite pests, utilizing biological enemies like macropredators and predatory mites, and especially phytoseiid mites (Phytoseiidae).

Predatory mites constitute an important group owing to their potential in controlling mite pest populations below the economic injury levels. Among various groups of pests, mites of the families Tetranychidae, Eriophyidae, Tenuipalpidae and Tarsonemidae constitute the most known preys of phytoseiid mites. The objective of using phytoseiids as biocontrol agents is to restore and/or to enhance the relationships between pests and their natural enemies either by reintroduction and or by creating the same habitat conditions under which the relationship would be strengthened (Collyer, 1956; Chant, 1959; McMurtry and Croft, 1997). Phytoseiidae is a large family with worldwide distribution and comprising about 1600 species belonging to over 70 genera. This family consists of three subfamilies, Amblyseinae, Phytoseiinae and Typhlodrominae. Effective biocontrol agents occur in all these three subfamilies. They have

drawn attention of economic entomologists and acarologists all over the world and have encouraged intensive and extensive faunistic studies. As a result, many countries have started implementing biological control programmes also as a part of IPM through mass rearing, release and export of phytoseiid predators. Phytoseiids enjoy a wide range of habitats, ranging from the arctic to the tropics. They could be found out from all types of plants comprising herbs, shrubs, trees, grasses, fungi, mosses and from any part of the plant, inflorescence, leaves, flowers etc. The ability to prosper on non-animal food items like pollen, honey and nectar is another factor behind their success as biocontrol agents. Besides these, phytoseiid mites possess an array of supreme adaptive features which often raise them to the level of potential predators of pest mites and also insects to a certain extent. These include wide distribution, high abundance, short life cycle than that of their prey, equivalent reproductive potential, good searching capacity, good dispersal rate, ability to survive on a low prey density, and adaptability to different ecological niches (McMurtry, 1982).

### **Role of phytoseiid mites**

Since phytoseiid mites are the most important biological control agents of phytophagous mites, numerous investigations have been conducted focusing on their development and reproduction (McMurtry *et al.*, 1970; McMurtry, 1982; Helle and Sabelis, 1985b). Different studies have been conducted to calculate their life history traits and life table parameters that could delineate their role in field conditions. These studies have considered phytoseiid species common in European agro-ecosystems such as *Kampimodromus aberrans* (e.g. Schausberger, 1997, 1998 a, 1998b; Pappas *et al.*, 2005; Broufas *et al.*, 2007; Kasap, 2005; Lorenzon *et al.* 2012), *Amblyseius andersoni* (e.g. Overmeer and Van Zon, 1982; Duso *et al.*, 1991; Zhang and Croft, 1994; Pozzebon and Duso 2008; Pozzebon *et al.*, 2009; Lorenzon *et al.*, 2012) and *Typhlodromus pyri* (Overmeer and Van Zon, 1982; Engel and Ohnesorge 1994; Zemek *et al.*, 1997; Schausberger, 1998a, 1998b, 1999a, 1999b; Pozzebon and Duso 2008; Lorenzon *et al.*, 2012).

On the basis of demographic parameters related to food preferences, some authors have proposed a classification of phytoseiids according to their diets (McMurtry *et al.*, 1997). In particular, they are classified as type I if specialized on species of the genus *Tetranychus*: this is the case of *Phytoseiulus persimilis* (Athias-Henriot). Type II includes *Galendromus occidentalis* (Nesbitt) and species of the genus *Neoseiulus* that prefer tetranychid prey. Phytoseiids of the type III, such as *A. andersoni*, *K. aberrans*, *T. pyri*, or *Phytoseius finitimus* Ribaga are polyphagous. Finally, predators of type IV like *Euseius finlandicus* (Oudemans) prefers pollen to prey. Phytoseiid belonging to type III or IV (eg. *A. andersoni*, *T. pyri*, *K.*

*aberrans*, *P. finitimus* and *E. finlandicus*) greatly predominate in orchards and vineyards in Europe and elsewhere, Phytoseiids proved to be more efficient in controlling phytophagous mites as compared to other predators which is attributable to certain characteristics such as: a) rapid development period (equal to or shorter than that of the prey), b) good prey searching ability, c) ability to consume plenty of prey and capability to survive even in food shortage.

### **Phytoseiid mites in orchards and vineyards in northern Italy**

A number of investigations of mite fauna have been carried out in vineyards (e.g., Duso and Liguori, 1984; Lozzia *et al.*, 1990; Zandigiacoimo *et al.*, 1992; Duso *et al.*, 1993) and fruit orchards (e.g. Ioriatti and Mattedi, 1988; Oberhofer *et al.*, 1985; Duso and Sbrissa, 1990) of northern Italy. A short description will be given for the most common species and most important in biological control: *Kampimodromus aberrans* (Oudemans), *Amblyseius andersoni* (Chant), *Typhlodromus pyri* (Scheuten) and *Phytoseius finitimus* Ribaga.

#### ***Kampimodromus aberrans***

*Kampimodromus aberrans* can be found as dominant in neglected fruit orchards but is rare in commercial orchards due to its susceptibility to different pesticides. The persistence of *K. aberrans* in vineyards seems positively influenced by the reduced use of insecticides and fungicides (Ivancich Gambaro, 1973, Tirello, 2012). *K. aberrans* proved to be effective in controlling spider mites (*Panonychus ulmi* (Koch) and *Eotetranychus carpini* (Oudemans)) in vineyards (Ivancich Gambaro, 1973, Duso *et al.*, 1983; Girolami, 1987; Duso, 1989). Recently, the role of this predatory mite has been evaluated in commercial apple orchards where a strain resistant to organophosphates was successfully released (Duso *et al.*, 2009). The life history and life-table parameters of this species reveal a preference for pollen and eriophyoid mites compared to spider mites (Schausberger, 1991, 1992; Lorenzon *et al.*, 2012). Its persistence on alternative foods in condition of prey scarcity is a key factor to prevent phytophagous mite outbreaks (Duso *et al.*, 2012).

#### ***Amblyseius andersoni***

*Amblyseius andersoni* was found to be a dominant species in apple orchards of northern Italy (Ioriatti *et al.*, 1988; Duso and Sbrissa, 1990) probably because of its resistance to many insecticides and fungicides (Ivancich Gambaro, 1975; Baillod *et al.*, 1985; Duso *et al.*, 1992; Angeli *et al.*, 1994). Its aggressiveness in interspecific predation can also explain the dominance within predatory mite communities (Croft, 1994; Schausberger and Croft, 2000). The life history and life-table parameters of this species show a high polyphagy (Dicke and

De Jong, 1988; Pozzebon and Duso, 2009; Lorenzon *et al.*, 2012). *Amblyseius andersoni* population dynamics sometimes shows unpredictable trends partly explained by adverse climate and food scarcity (Duso *et al.*, 2012).

### ***Typhlodromus pyri***

*Typhlodromus pyri* has been reported as the most important predatory mite in European apple orchards (Collyer, 1956, 1964; Johnsen and Hanssen, 1986; Duso and Sbrissa, 1990) and vineyards (Schruft, 1985; Duso, 1989; Engel and Ohnesorge, 1994). Pesticide resistant strains have been widely detected (Duso *et al.*, 1992; Bonafos *et al.*, 2008). *Typhlodromus pyri* populations can persist for long time in conditions of prey scarcity by exploiting alternative foods such as pollen and fungi (e.g., Addison *et al.*, 2000; Engel and Ohnesorge, 1994; Zemek *et al.*, 1997, Pozzebon and Duso, 2009). It proved to be competitive towards other specialist and generalist predatory mites (Croft and McRae, 1992a, 1992b; Schausberger, 1997, 1998, 1999a, 1999b). However, the successful colonization of vineyards by *T. pyri* can be hindered by climatic conditions and cultivar features (Duso and Pasqualetto, 1993; Duso and Vettorazzo, 1999).

### ***Phytoseius finitimus***

*Phytoseius finitimus* is another phytoseiid species recorded frequently in European vineyards and apple orchards (Castagnoli, 1989; Nicotina, 1996; Papaioannou-Souliotis *et al.*, 1999; Kreiter *et al.*, 2000; Ragusa and Tsolakis, 2001). It has been found to be more abundant on grape varieties with pubescent leaf undersurfaces (Duso and Moretto, 1994; Duso and Vettorazzo, 1999). Its food range includes tetranychids, eriophyoids and pollen (Rasmy and El-Banhawy, 1975). This species have potential for controlling *P. ulmi* but seems to be ineffective towards *E. carpini* and proved to be susceptible to various pesticides (Duso and Vettorazzo, 1999).

## **Factors affecting phytoseiid mite performance**

The spread, colonization, and the persistence of phytoseiid mites on different crops are affected by many factors. Environmental factors exert major pressure on a large scale. Phytoseiid species even in fruit and vine growing areas have a different distribution according to altitude. For example, *T. pyri* is found most frequently in hilly areas (Mathis, 1958). Temperature strongly affects the development and reproduction of predatory species used as biocontrol agents. The intrinsic rate of natural increase ( $r_m$ ) is an important parameter for assessing the reproductive potential of a predator under laboratory conditions, and



temperature is a significant determinant of this parameter (Sabelis, 1985; Janssen and Sabelis, 1992; Roy *et al.*, 2003). Among abiotic factors important for establishment and efficacy of introduced and native phytoseiid mites, relative humidity is probably second to temperature (Sabelis, 1985). Different studies showed the effects of low relative humidity on the biology and performance of phytoseiids (Sabelis, 1985; Zhang and Kong, 1985, Van Dinh *et al.*, 1988; Mangini and Hain, 1991). For example, *K. aberrans* egg-hatching is more affected by low humidity than that of *T. pyri* and *E. finlandicus* (Schausberger 1998). This phenomenon may contribute to explaining why this species is attracted to hairy leaves, with their inherent lower risk of egg desiccation. Many phytoseiids have low rates of egg eclosion below 50% relative humidity.

Other factors such as predator-prey relationships, interspecific competition and pest management program are equally important in establishment and colonization of phytoseiid mites (Croft and McRae, 1992a, 1992b; Croft *et al.*, 1992). Differences in plant characteristics may further affect the spread and the establishment of phytoseiid mite species in orchards (Chant, 1959; Collyer 1956; Blommers and Overmeer, 1986; Duso *et al.*, 2003) and vineyards (Duso, 1992; Camporese and Duso, 1996). A number of predatory mites preferred to colonize pubescent leaf undersurfaces (Kreiter *et al.* 2002) and this phenomenon could affect interspecific competition in vineyards (Duso and Vettorazzo, 1999) and apple orchards (Duso *et al.*, 2009). Pollen and fungal spores, important alternative foods for generalists, can be captured and retained easily by leaves having numerous trichomes or domatia (Kreiter *et al.* 2002; Roda *et al.*, 2003). Leaf trichomes and/or domatia provide refuge for phytoseiid mites from their predators (Roda *et al.*, 2000; Norton *et al.*, 2001). In addition, a high trichome density can improve micro-environmental conditions for phytoseiids (Grostal and O'Dowd 1994).

The persistence and effectiveness of generalist phytoseiid mites is based on their ability to survive in conditions of prey scarcity and the use of alternative food sources. It is well known that generalist phytoseiids can develop and reproduce on pollen, but the impact of pollen on phytoseiid populations in vineyards has only been studied at a small spatial and short temporal scale. Studies showed that *T. pyri* populations peaked following phases with large pollen availability on leaves (Engel and Onhesorge, 1994b). Long-term studies in north-eastern Italy confirmed a similar relationship for *T. pyri*, *K. aberrans* and, to a lesser extent, *A. andersoni* (Duso *et al.*, 1997). Grape pathogenic fungi can also play an important role as alternative foods for generalist phytoseiids (Pozzebon and Duso 2009). Late-summer spread of grape downy mildew (GDM) foliar symptoms has been associated with sudden population increases

of *A. andersoni* and, to a lesser extent, *T. pyri* (Duso *et al.*, 2003). Additional effects of GDM involved competition between *A. andersoni* and *T. pyri*: GDM provided advantage to *A. andersoni* over *T. pyri* that was outcompeted by the former species. An interesting case of interactions between predators and prey mediated by GDM concerned the phytoseiid *Paraseiulus talbii* (Athias-Henriot) and the tydeid *Tydeus caudatus* Dugès. The latter can develop and reproduce on GDM and is the preferred prey for *P. talbii* (Camporese and Duso, 1995). GDM positively affected tydeid populations and consequently *P. talbii* numbers (Duso *et al.*, 2005).

Competition influence predatory mite community structure. Generalist phytoseiids feed more on other phytoseiids than specialists do (Croft and Croft 1996, Croft *et al.*, 1996). Implications of intraspecific competition for predatory mite population dynamics have been widely discussed (Zhang and Croft, 1995a; Schausberger and Croft, 2000). Predation by large phytoseiids or by macropredators, such as mites in the Anystidae, Cheyletidae, Erythraeidae, Cunaxidae, and Bdellidae, as well as insects and spiders, is another factor to be considered. Some phytoseiids use domatia to escape detection; some use avoidance behavior when contacted. The generalist *T. pyri* spends much time in cover on leaves, is very mobile, and shows evasive behavior when contacted by predators (Croft and Croft, 1996). In contrast, adult females of the specialist *G. occidentalis* show little if any avoidance behaviors and they are easily captured and consumed even by only slightly larger phytoseiids. Immature stages differ greatly in activity, food requirements, feeding habits, and tendencies for intra- and interspecific predation. Specialists have immatures that seem to develop more rapidly and require less food (especially Type II species), but they are less active in intra- and inter-specific predation than immatures of generalists (Croft and Croft, 1993; Zhang and Croft, 1995; Schausberger and Croft, 2000).

### **Effects of pesticides on phytoseiids**

Chemical control is a commonly used management tactic against different pest species of tetranychids in several crops of economic importance. However the intensive use of pesticides has compromised their effectiveness, especially due to the evolution of pesticide resistance to the main active ingredients (Cranham and Helle, 1985; Nauen *et al.*, 2001). Compatibility of pesticides with beneficial arthropods is a key aspect of Integrated Pest Management (IPM). The use of selective pesticides that do not harm phytoseiids is needed. It has been observed that continuous application of non selective pesticides may interfere with phytoseiid mite performance because these predators are generally more susceptible to pesticides than their prey (Croft, 1990). Traditionally, the measurement of acute toxicity of pesticides to beneficial

arthropods has relied largely on the determination of an acute median lethal dose or concentration. However, the estimated lethal dose during acute toxicity tests may only be a partial measure of deleterious effects. In addition to direct mortality induced by pesticides, their sub-lethal effects on arthropod physiology and behavior must be considered for a complete analysis of their impact. Pesticides applied in controlling pests and diseases can exert pronounced effects on phytoseiid survival, development and reproduction and may alter their response to different factors. These pesticides may also have strong, negative, direct and indirect impacts on a broad range of non-target organisms. So the use of pesticides in IPM systems requires a prior assessment of their possible side effects (lethal and sub-lethal) on beneficials. Sub-lethal effects are defined as effects (either physiological or behavioral) on individuals that survive exposure to a pesticide. These sub-lethal effects can be categorized as physiological effects on development, adult longevity, fecundity and sex ratio and behavioral effects like mobility, orientation, feeding behavior.

For the past 20 years, the effects of pesticides on beneficial arthropods have been the subject of an increasing number of studies and several laboratory and field studies have been conducted to evaluate the side effects of pesticides on predators belonging to family Phytoseiidae. In European vineyards, predatory mites are exposed to fungicides and insecticides. The latter are required to control berry moths, leafhoppers and minor pests. Studies conducted in the last decades showed the detrimental effects of a number of pesticides (e.g. organophosphates, carbamates and pyrethroids) on predatory mites (e.g. Marchesini 1989; Duso *et al.*, 1992). It has been observed that certain chemicals may not have direct effect on predatory mites in terms of mortality but are associated with some sub-lethal effect like reduction in fecundity rate, and repellent effects that ultimately results into overall population decrease of predatory mites. Reduction in fecundity of predatory mites associated with pesticides may be due to both physiological and behavioral effects. Some experiments showed that despite negligible mortality and high resistance to some OPs and carbamate insecticides in three *A. andersoni* strains from north Italian orchards and vineyards, the fecundity of the adult females was sometimes significantly reduced (Duso *et al.*, 1992). Tirello (2010) evaluated lethal and sub-lethal effects of pesticides on predatory mite females. Spinosad and etofenprox were associated with significant effects on mite survival and fecundity. Thiamethoxam caused a significant reduction in fecundity. Duso *et al.* (2008) tested spinosad at the maximum rate allowed in Italy (330 ppm) and found 47% of mortality of *P. persimilis* females along a 57% of reduction in oviposition. Another sublethal effect associated with pesticides is the functional response of predatory mites. Pesticides like

imidacloprid can significantly affect the functional response of predatory mite *Neoseiulus californicus* (Poletti *et al.*, 2007), with a conspicuous increase in handling time by the predator during the process of prey identification, capture, attack, consumption and digestion and a decrease in predators' attack coefficient. Alteration in these parameters led to a reduction of approximately 55% in the consumption of *T. urticae* eggs by this predator. Castagnoli *et al.* (2005) observed that even though imidacloprid showed low toxicity on adult *N. californicus* females, it significantly reduced the predator's fecundity. Therefore a reduction in predatory capacity of predatory mites on a contaminated prey may affect its reproductive capacity, inhibiting its population growth and consequently affecting its performance in mite-pest management programmes.

Applications of certain insecticides may also result in sublethal effects on reproduction, foraging behavior, fecundity and longevity (Croft, 1990) For example; foliar residues (both wet and dry) may inhibit volatile cues emitted by hosts, which are used by certain natural enemies to detect host location within plants (Dicke and LEM, 1999; Gohole *et al.*, 2003). This may influence foraging behavior and the time required to find hosts. Sub-lethal effects may also inhibit the ability of natural enemies to establish populations, suppress the ability of natural enemies to utilize a host, impact parasitism (for parasitoids) or consumption (for predators) rates, decrease longevity and progeny production rate, reduce host availability, inhibit ability to recognize hosts and influence the sex ratio (females: males) (Rosenheim and Hoy 1988; Grafton-Cardwell *et al.*, 2006). Natural enemies feeding on plant pollen or nectar may also ingest the active ingredient. Furthermore, any repellent properties, based on vapor activity or volatility, may prevent natural enemies from entering or re-colonizing habitats; however, any behavioral effects that may be observed are likely due to detergency and sublethal effects associated with direct exposure to the active ingredient (Croft, 1990).

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## **Chapter II**

### **Augmentative releases of the predatory mite *Kampimodromus aberrans* in organic and conventional apple orchards**

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Submitted to Crop Protection as:

Shakeel Ahmad, Alberto Pozzebon, Carlo Duso - Augmentative releases of the predatory mite *Kampimodromus aberrans* in organic and conventional apple orchards

I collected most of the data, contributed to statistical analysis and drafted a manuscript

## Abstract

Experiments of the release of the predatory mite *Kampimodromus aberrans* were conducted in organic and conventional apple orchards located in North-eastern Italy. Releases were made in 2010 and 2011 following a completely randomized design and observations were carried out from 2010 to 2012. The material utilized for releases was collected from a commercial vineyard where *K. aberrans* was the dominant phytoseiid species. The most frequent insecticides used in organic orchards were pyrethrins and spinosad, whereas neonicotinoids, OPs and IGRs were mostly used in conventional orchards. Predatory mites were significantly higher in released plots as compared to control plots and *K. aberrans* populations were higher in organic compared to conventional orchards. In 2010, no effect of *K. aberrans* release and orchard management was observed on populations of the native predatory mite *Amblyseius andersoni*. However, in 2011 *A. andersoni* population densities were lower in released than in control plots. In conventional orchards *K. aberrans* did not establish probably because of a series of non-selective insecticide and fungicide treatments. In one experimental site releases were evaluated on two organic orchards (Florina and Golden Delicious) managed with the same cropping systems. In both 2010 and 2011 releases, *K. aberrans* population densities were higher in release than control plots both in Florina and Golden Delicious cultivars. On Florina *K. aberrans* population appeared to be larger than on Golden Delicious suggesting a role of leaf morphology in predatory mite colonization. On Golden Delicious, *A. andersoni* population densities were lower in released than in control plots. Implications for mite management in organic and conventional orchards are discussed.

## Introduction

Phytophagous mite densities in apple orchards are kept within economic levels by a complex of predators comprising macropredators and predatory mites (McMurtry *et al.*, 1970; Van de Vrie, 1985; Blommers, 1994; Solomon *et al.*, 2000). Macropredators exert a significant role in reducing large mite populations but their persistence declines at low prey densities. Predatory mites occurring in apple orchards are less voracious than macropredators but can persist longer in conditions of prey scarcity. Those belonging to the Phytoseiidae family are generalist predators that survive and reproduce by feeding on non-prey food such as pollen, honeydew and fungi (McMurtry, 1992; McMurtry and Croft, 1997; Schausberger, 1991; Pozzebon and Duso, 2008). They are crucial in preventing phytophagous mites' outbreaks. The most important phytoseiid species recorded in European apple orchards are *Typhlodromus pyri* Scheuten, *Euseius finlandicus* (Oudemans) and *Amblyseius andersoni*

(Chant); *Kampimodromus aberrans* (Oudemans) and *Phytoseius finitimus* Ribaga are also common in some regions (e.g. Collyer, 1964; Ivancich Gambaro 1975; El Borolossy and Fischer-Colbrie, 1989a, 1989b; Duso and Sbrissa, 1990; Schausberger, 1998a). Several factors can affect the success of predatory mites as biological control agents but the most relevant are pesticide application, climatic conditions, food availability, cultivar features and intraguild competition (e.g. Van de Vrie, 1985; Duso, 1992a; Blommers, 1994; Croft, 1994; Cross and Berrie, 1994, 1996; Schausberger, 1998b; Solomon *et al.*, 1993, 2000; Fernandez *et al.*, 2006; Bonafos *et al.*, 2007; Duso *et al.*, 2012). In biological control programmes these factors and predatory mite species attributes should be considered to maximize their performance and preserve their populations in the long term (Croft, 1994; Croft and Luh, 2004). Knowledge on the mechanisms involved in the coexistence of different species and the displacement of one species by a more competitive one are also crucial in biological control tactics (e.g. Croft and Croft, 1993; Zhang and Croft, 1995; Schausberger, 1997, 1998b; Rosenheim *et al.*, 1995).

Releasing predatory mites proved to be a successful technique in European vineyards where *T. pyri* and *K. aberrans* were the most commonly used species (Boller, 1978; Girolami, 1987; Ivancich Gambaro, 1987; Duso, 1989). *K. aberrans* was the most competitive predator when released with *A. andersoni* and *T. pyri* (Duso, 1989). The competition between *T. pyri* and *A. andersoni* was sometimes mediated by grape variety (Duso and Vettorazzo, 1999). The possibility of releasing predatory mites in apple orchards has also been explored and some experiments have obtained good results (e.g., Bailod and Guignard, 1984; Marshall *et al.*, 2001).

*K. aberrans* has been proved successful in controlling phytophagous mites in vineyards but this capacity has often been limited by the use of non-selective pesticides, mainly ethylene-bis-dithiocarbamate (EBDC) fungicides and organophosphates (OPs) (Ivancich Gambaro, 1973; Girolami, 1987). Then resistant strains have been detected and released in vineyards and apple orchards (see Duso *et al.*, 2009 and Tirello *et al.*, 2012 for details). In some regions *K. aberrans* has been commonly found in apple orchards but little has been reported about pesticide pressure in these situations (El Borolossy and Fischer-Colbrie 1989a, 1989b; Schausberger, 1991; Nicotina and Cioffi, 1998; Cobanoglu and Ozman, 2002; Kasap, 2004). It should be stressed that a high number of insecticide applications are made annually in conventional apple orchards where predatory mites able to persist are usually characterised by some resistance. *A. andersoni* and *T. pyri* have been reported to be resistant to various pesticides (e.g., OPs, carbamates, pyrethroids) and are known to be widely distributed in



European apple orchards since the 1970s (e.g., Ivancich Gambaro, 1975; Anber and Overmeer, 1988; Duso *et al.*, 1992; Fitzgerald and Solomon, 1992).

The impact on predatory mites of pesticides applied in organic orchards has not been investigated in depth. In this study an OP resistant strain of *K. aberrans* was released in conventional and organic apple orchards naturally colonized by *A. andersoni*. The establishment of *K. aberrans* under different orchard management and interactions between native and released predatory mites were assessed for three years.

## **Materials and Methods**

### **Experimental orchards**

Experiments were conducted in the Veneto region, North-eastern Italy from 2010 to 2012. In 2010, five sites (Pernumia, Lancenigo, Povegliano 1 and 2, and Spresiano) were identified for predatory mite releases (Table 1). Three organic orchards were considered at Pernumia (Golden Delicious) and Spresiano (two contiguous orchards with Golden Delicious and Florina cultivars), and three conventional orchards in the remaining sites (Lancenigo, Povegliano 1 and 2) the latter containing Golden Delicious. Golden Delicious is characterized by a glabrous leaf under-surface, a relatively low number of domatia at the conjunction of main veins and a low hair density along the veins (Duso *et al.*, 2009). Florina shows a moderately pubescent leaf under-surface, a relatively high number of domatia at the conjunction of main veins and high hair density along the veins (Duso *et al.*, 2003).

In 2011, three organic orchards with Golden Delicious cultivar were selected for releases at Spresiano, Pernumia and San Pietro Viminario and three conventional apple orchards at Lancenigo and Povegliano (1 and 2). At Spresiano, there were additional releases in the Florina orchard.

The most frequent insecticides used in organic orchards were pyrethrins and spinosad, whereas various formulations were used in conventional orchards (Table 1). It should be stressed that some fungicides known for their negative effects on predatory mites (e.g. lime-sulphur, mancozeb, pyrimethanil) were also applied in conventional orchards.

### **Experimental design**

The effect of predatory mite releases was tested with a non-release treatment (control) following a completely randomized design. In each orchard, predatory mites were released in four plots (replicates) containing four apple trees, separated by 8-10 trees in the subsequent plots. In 2010, *K. aberrans* were released in June. The material utilized for releases (shoots)

was collected from a commercial vineyard located at Monteforte d'Alpone (Verona province), where *K. aberrans* was the dominant phytoseiid species. This strain proved to persist despite the use of several pesticides, namely OPs and EBDCs (Posenato, 1994). In June 2010, before releases, 50 shoots (10-15 leaves/shoot) were collected from the source vineyard and taken to the laboratory for analysis using a stereomicroscope. The identity and density of phytoseiids were assessed and the presence of other arthropods was recorded. A mean density of 5.01 *K. aberrans* motile forms per shoot was calculated. This figure was considered in order to assess the number (approximately 50) of predatory mites to be released per tree. Shoots were placed along the main branches inside the canopy.

In 2011, releases were in February and June. In February, prior to release, 50 two-year-old branches were collected from the vineyard and analyzed using a stereomicroscope. A mean density of 4.05 overwintered females per internode was recorded. Approximately 100 females of *K. aberrans* were released per plant via two-year-old branches using procedures described in Duso (1989). In June, densities of *K. aberrans* were calculated on shoots removed from the same vineyard. A mean of 20.08 *K. aberrans* per shoot was calculated. Approximately 200 *K. aberrans* were released per plant by using shoots and following previous procedures.

For the 2010 trials sampling was conducted from August to September and in spring 2011. The positive results obtained with releases at Spresiano suggested prolonging samplings in these orchards for three years (2010-2012). In the 2011 trials, observations were conducted during the vegetative season and in spring 2012. Additional observations were made in 2012 at Spresiano only. Seasonal mite abundance (mainly Tetranychidae, Eriophyidae, Tydeidae and Phytoseiidae) was monitored by taking 25 leaves per replicate (100 leaves per treatment) per sampling date. Leaves were transferred to the laboratory and immediately analyzed using a stereomicroscope in order to assess the identity and density of mites.

### **Statistical analysis**

Data collected during the year when *K. aberrans* was released (2010 and 2011) were analyzed using a Restricted Maximum Likelihood (REML) repeated measures model with the Proc MIXED of SAS (SAS Institute Inc., 1999). *K. aberrans* release, orchard type, time of sampling and their interactions were considered as source of variation for *K. aberrans* and *A. andersoni* populations and *F* tests were used to evaluate their effects ( $\alpha = 0.05$ ). Degrees of freedom were estimated using the Kenward–Roger method (Littell *et al.*, 1996). According to Aikake's Information Criterion, first-order autoregressive proved to be the best fitting covariance structure for correlating different sampling dates (Littell *et al.*, 1996). Differences among treatments were evaluated with a t-test ( $\alpha = 0.05$ ) to least square means. The SLICE

option of the LSMEANS statement was used to test *K. aberrans* release and orchard type effect variation during observation periods (SAS Institute Inc., 1999). Data collected in the spring after release (i.e., spring 2011 and 2012) were analysed with a REML model with the Proc MIXED of SAS (SAS Institute Inc., 1999). *K. aberrans* release, orchard type, time and their interactions were considered as source of variation for *K. aberrans* and *A. andersoni* populations.

Three years of data collected in Spresiano were also analyzed using a REML repeated measures model. In this model *K. aberrans* release, year, time of observation nested within year and their interactions were considered as source of variation for *K. aberrans* and *A. andersoni* populations. Degrees of freedom were estimated using the Satterthwaite approximation method (Littell *et al.*, 1996). First-order autoregressive was used as covariance structure for correlating different sampling dates (Littell *et al.*, 1996). Differences among treatments were evaluated with a t-test ( $\alpha = 0.05$ ) to least square means.

Mite densities were analyzed separately. All data were checked for normality assumption and thus the number of phytoseiids per leaf was  $\log(x + 1)$  transformed.

## Results

### Mite population dynamics in orchards (2010 releases)

Among native predatory mites, *A. andersoni* was found in all orchards at low to moderate densities. Eriophyoids (i.e. *Aculus schlechtendali* Nalepa) were observed at low population densities and tetranychids (i.e. *Panonychus ulmi* (Koch) occurred at negligible levels. Tydeids were also recorded on most sampling dates but at low densities. The effect of *K. aberrans* releases in conventional and organic apple orchards is reported with regard to *K. aberrans* and *A. andersoni* abundance.

#### *Kampimodromus aberrans*

*K. aberrans* releases were successful and predatory mite numbers were much higher in the release than in the control plots ( $F_{1, 83.8} = 21.45$ ;  $P < 0.0001$ , Figure 1). Orchard management influenced the success of releases ( $F_{1, 95.9} = 10.53$ ;  $P = 0.002$ ). In organic orchards *K. aberrans* populations were larger in the release than in the control plots ( $t_{83.8} = 5.66$ ;  $P < 0.0001$ ) while there were no differences in conventional orchards ( $t_{83.8} = 0.98$ ;  $P = 0.328$ ) due to low predatory mite numbers (Figure1). An interaction was observed between time and *K. aberrans* release ( $F_{3, 146} = 6.71$ ;  $P = 0.0003$ ) as the variation in predatory mites population level over time was observed in release plots ( $F_{3, 146} = 12.72$ ;  $P < 0.0001$ ) but not in control plots ( $F_{3, 146}$

= 0.04;  $P = 0.985$ ). *K. aberrans* population variation over time was also influenced by orchard management ( $F_{3, 146} = 4.02$ ;  $P = 0.009$ ), being significant in organic ( $F_{3, 83.8} = 32.06$ ;  $P < 0.0001$ ) but not in conventional orchards ( $F_{3, 83.8} = 0.97$ ;  $P = 0.328$ ). Interaction among *K. aberrans* release, orchard management and time was also significant ( $F_{7, 157} = 32.06$ ;  $P < 0.0001$ ) since the effect of *K. aberrans* release\*time was significant in organic ( $F_{7, 157} = 4.56$ ;  $P = 0.004$ ) but not in conventional orchards ( $F_{7, 157} = 0.37$ ;  $P = 0.918$ ).

In the sampling performed in spring 2011 *K. aberrans* was found only in one organic orchard (Spresiano) and no effects of *K. aberrans* release ( $F_{1, 43.7} = 3.17$ ;  $P = 0.081$ ) or orchard management ( $F_{1, 3.4} = 1.25$ ;  $P = 0.336$ ) were observed.

### ***Amblyseius andersoni***

No effects of *K. aberrans* release and orchard management were observed on *A. andersoni* populations (respectively  $F_{1, 68.1} = 1.94$ ;  $P = 0.168$  and  $F_{1, 3.03} = 5.23$ ;  $P = 0.105$ ; Fig. 2). The same trend was observed in the sampling done in spring 2011 (respectively  $F_{1, 42.9} = 1.69$ ;  $P = 0.20$  and  $F_{1, 2.72} = 0.08$ ;  $P = 0.792$ ).

### ***Aculus schlechtendali***

In 2010 no effects of *K. aberrans* release and orchard management were observed on apple rust mite densities ( $F_{1, 84} = 0.72$ ;  $P = 0.398$ ;  $F_{1, 2.87} = 0.10$ ;  $P = 0.774$ , respectively). The same trend was observed in the sampling done in spring 2011 ( $F_{1, 193} = 0.68$ ;  $P = 0.411$  and  $F_{1, 2.87} = 0.10$ ;  $P = 0.771$ ; respectively).

## **Mite population dynamics in orchards (2011 releases)**

Among phytoseiid mites *A. andersoni* and *K. aberrans* occurred at low to moderate population densities while phytophagous mite numbers were negligible. The effects of *K. aberrans* releases in organic and conventional orchards are reported below.

### ***Kampimodromus aberrans***

*K. aberrans* densities were confirmed to be higher in the release plots than in control plots ( $F_{1, 113} = 13.08$ ;  $P = 0.0004$ ). Populations appeared to reach lower levels than in the previous experiment despite multiple releases (Figure 3). Orchard management did not affect predatory mite densities ( $F_{1, 4} = 0.32$ ;  $P = 0.604$ ). There was a variation in *K. aberrans* population densities among sampling dates ( $F_{5, 224} = 5.39$ ;  $P = 0.0001$ ) but this effect was significant only in organic orchards ( $F_{5, 224} = 5.71$ ;  $P < 0.0001$ ). Other interactions were not significant.

In spring 2012 there were more *K. aberrans* in the release plots than in control plots ( $F_{1, 42} = 12.60$ ;  $P = 0.001$ ). No effects of orchard management nor of interaction *K. aberrans*

release\*orchard management were observed ( $F_{1, 3.99} = 0.07$ ;  $P = 0.806$ ;  $F_{1, 42} = 0.15$ ;  $P = 0.701$ , respectively).

### ***Amblyseius andersoni***

*K. aberrans* releases determined a significant effect on *A. andersoni* populations ( $F_{1, 103} = 8.24$ ;  $P = 0.005$ ; Fig. 4). There was no significant effect of orchard management on *A. andersoni* ( $F_{1, 4.04} = 4.63$ ;  $P = 0.09$ ) nor of the interaction *K. aberrans* release\*orchard management ( $F_{1, 103} = 0.71$ ;  $P = 0.401$ ). A variation in *A. andersoni* population levels was observed among sampling dates ( $F_{5, 214} = 79.83$ ;  $P < 0.0001$ ). The interaction *K. aberrans* release\*time was significant ( $F_{5, 214} = 6.25$ ;  $P < 0.0001$ ) as *A. andersoni* declined in the release plots in the second part of summer (Figure 4). There was also a significant interaction time\*orchard management ( $F_{5, 214} = 11.92$ ;  $P < 0.0001$ ) as *A. andersoni* numbers were higher in conventional orchards in late summer only.

In the sampling conducted in spring 2012 there were no significant effects of release ( $F_{1, 42} = 2.15$ ;  $P = 0.149$ ), orchard management ( $F_{1, 3.98} = 2.60$ ;  $P = 0.182$ ) and their interaction ( $F_{1, 42} = 3.87$ ;  $P = 0.056$ ) on *A. andersoni*.

### **Mite population dynamics in Florina and Golden Delicious orchards (Spresiano, 2010 trials)**

Predatory mite releases were successful in the Spresiano orchards containing Florina and Golden Delicious cultivars and managed with the same cropping methods. Observations in these orchards lasted three years (2010-2012).

#### ***Kampimodromus aberrans* and *A. andersoni* in Florina orchard**

*K. aberrans* densities were higher in the release than in control plots ( $F_{1, 24.5} = 89.55$ ;  $P < 0.0001$ ) where predatory mites were recorded from late summer 2010 (Figure 5). The effect of release was not constant over the observation period ( $F_{10, 63.1} = 9.79$ ;  $P < 0.0001$ ). Higher numbers of *K. aberrans* were observed in the release plots compared to the control in August 2010, from late summer onwards in 2011 and during spring and summer in 2012 (Fig. 5). The population level of the predatory mite varied among sampling dates ( $F_{10, 63.1} = 33.72$ ;  $P < 0.0001$ ). Moreover, a significant interaction between *K. aberrans* release and years was observed ( $F_{2, 35.7} = 14.53$ ;  $P < 0.0001$ ). In release plots *K. aberrans* population increased over the three years (2010 vs. 2011:  $t_{33} = -2.47$ ;  $P = 0.018$ ; 2010 vs. 2012:  $t_{34.6} = -12.45$ ;  $P < 0.0001$ ; 2011 vs. 2012:  $t_{39.6} = -11.4$ ;  $P < 0.0001$ ) while in control plots they were similar in

2010 and 2011 ( $t_{34,6} = -2.01$ ;  $P = 0.053$ ) but higher in 2012 (vs. 2010:  $t_{33} = 5.73$ ;  $P < 0.0001$ ; vs. 2011:  $t_{39,6} = 4.44$ ;  $P < 0.0001$ ).

No effect of *K. aberrans* release was observed on *A. andersoni* populations in Florina orchard ( $F_{1, 28,6} = 0.04$ ;  $P = 0.845$ , Figure 6). *A. andersoni* population dynamics fluctuated at low levels over the three-year period ( $F_{2, 39,1} = 14.66$ ;  $P < 0.0001$ ) and among sampling dates within years ( $F_{10, 61,7} = 5.36$ ;  $P < 0.0001$ ).

### ***Kampimodromus aberrans* and *A. andersoni* in Golden Delicious orchard**

*K. aberrans* population densities were higher in release plots than in the control ( $F_{1, 28,2} = 127.60$ ;  $P < 0.0001$ ). The effect of release varied over sampling dates ( $F_{10, 60,2} = 14.97$ ;  $P < 0.0001$ ). In release plots there were higher numbers of *K. aberrans* in mid-summer 2010, August 2011 and late summer 2012 (Figure 7). The population level of the predatory mite varied among sampling dates ( $F_{10, 60,2} = 19.28$ ;  $P < 0.0001$ ), and a significant interaction *K. aberrans* release\*year was found ( $F_{2, 38,7} = 22.07$ ;  $P < 0.0001$ ). In release plots *K. aberrans* populations were higher in 2010 and 2012 compared to 2011 (respectively:  $t_{37,6} = 6.29$ ;  $P < 0.0001$ ;  $t_{42} = 6.69$ ;  $P < 0.0001$ ) but no differences were observed between 2010 and 2012 ( $t_{36,5} = 0.88$ ;  $P = 0.383$ ). In control plots the population levels were similar among the three years (2010 vs. 2011:  $t_{37,6} = 1.87$ ;  $P = 0.069$ ; 2010 vs. 2012:  $t_{36,5} = 0.99$ ;  $P = 0.327$ ; 2011 vs. 2012:  $t_{42} = 0.63$ ;  $P = 0.531$ ).

*A. andersoni* population size in Golden Delicious appeared to be higher than that observed in Florina orchard (Figure 8). Predatory mite levels were lower in *K. aberrans* release plots compared to control plots ( $F_{1, 28,5} = 29.59$ ;  $P < 0.0001$ ). This effect varied among years ( $F_{2, 39} = 8.84$ ;  $P = 0.001$ ). *A. andersoni* population was higher in the control than release plots in 2010 ( $F_{1, 34,2} = 18.47$ ;  $P = 0.0001$ ) and 2012 ( $F_{1, 38,9} = 12.11$ ;  $P = 0.001$ ), but not in 2011 ( $F_{1, 30,3} = 2.43$ ;  $P = 0.155$ ). Variations in *A. andersoni* numbers were observed among the three years ( $F_{2, 39} = 8.84$ ;  $P = 0.001$ ) and sampling dates within years ( $F_{10, 61,2} = 9.09$ ;  $P < 0.0001$ ).

### **Mite population dynamics in Florina and Golden Delicious orchards (Spresiano, 2011 trials)**

#### ***Kampimodromus aberrans* and *A. andersoni* in Florina orchard**

*K. aberrans* population levels were higher in release plots compared to control plots ( $F_{1, 20,5} = 26.10$ ;  $P < 0.0001$ ). Predatory mite population reached higher levels in 2012 than 2011 ( $F_{1, 29,6} = 229.34$ ;  $P < 0.0001$ ). The effect of release varied among sampling dates within years

( $F_{7, 43} = 6.60$ ;  $P < 0.0001$ ) resulting significant from August onwards in 2011, and in mid-summer 2012 (Figure 9).

*A. andersoni* population levels were similar among treatments ( $F_{1, 20.2} = 0.01$ ;  $P = 0.926$ ) and persisted at low levels in both years ( $F_{1, 28.9} = 2.02$ ;  $P = 0.166$ ). Population densities fluctuated among sampling dates within years ( $F_{7, 39.4} = 4.29$ ;  $P = 0.001$ , Figure10).

### ***Kampimodromus aberrans* and *A. andersoni* in Golden Delicious orchard**

*K. aberrans* population densities appeared to be lower in Golden Delicious than in Florina orchard (Figure11). They were higher in release plots compared to the control ( $F_{1, 17.9} = 28.48$ ;  $P < 0.0001$ ). A significant interaction *K. aberrans* release \* year was found ( $F_{1, 28.2} = 19.78$ ;  $P < 0.0001$ ). Population level increased from 2011 to 2012 in release plots ( $F_{1, 28.2} = 32.52$ ;  $P < 0.0001$ ) but not in control plots ( $F_{1, 28.2} = 0.35$ ;  $P = 0.562$ ). A significant interaction *K. aberrans* release \* sampling time was found ( $F_{7, 43.9} = 5.09$ ;  $P < 0.001$ ) as *K. aberrans* population fluctuated in release plots ( $F_{8, 44.3} = 12.82$ ;  $P < 0.0001$ ) but not in control plots ( $F_{8, 44.3} = 1.01$ ;  $P = 0.441$ ).

*Amblyseius andersoni* numbers were lower in release plots ( $F_{1, 25.1} = 33.04$ ;  $P < 0.0001$ ) but this effect varied between years ( $F_{1, 33.9} = 31.14$ ;  $P < 0.0001$ , Figure12). *A. andersoni* population was larger in control plots compared to release plots in 2012 ( $F_{1, 31.7} = 49.69$ ;  $P < 0.0001$ ) but not in 2011 ( $F_{1, 25.6} = 0.06$ ;  $P = 0.803$ ). Variation was also observed among sampling dates within years ( $F_{17, 42.1} = 3.99$ ;  $P = 0.002$ ).

## Discussion

In Europe *K. aberrans* is commonly recorded in neglected apple orchards but less frequently in commercial orchards (Genini *et al.*, 1983; El-Borolossy and Fischer-Colbrie, 1989a, 1989b; Duso and Sbrissa, 1990; Costa-Comelles *et al.*, 1990; Espinha *et al.*, 1998; Baudry *et al.*, 1999; Minãrro *et al.*, 2002; Fitzgerald and Solomon, 2002). Detailed observations made in the Trentino-Alto Adige region, where most Italian apple production is concentrated, confirmed similar patterns (Oberhofer and Waldner, 1985; Ioriatti and Mattedi, 1988; Solva *et al.*, 1997). The susceptibility of *K. aberrans* to various pesticides can largely explain this situation (Fauvel and Gendrier, 1992). The discovery of *K. aberrans* strains showing tolerance to OPs and EBDCs in Italian vineyards (Posenato, 1994) suggested their release in some commercial orchards located in Trentino. These experiments were successful despite the use of various pesticides and competition with native predatory mites (Duso *et al.*, 2009). More recently the resistance of *K. aberrans* strains to OPs was shown in the laboratory (Tirello *et al.*, 2012) and additional studies were performed to evaluate lethal and sub-lethal effects of various insecticides on one of these strains. The latter study showed that OPs, IGRs and neonicotinoids caused low effects on survival of *K. aberrans* females but reduced their fecundity with potential implications for persistence in apple orchards (Duso *et al.*, submitted). Apple orchards in northern Italy are usually inhabited by *A. andersoni* and less commonly by *T. pyri* and *E. finlandicus* (Duso and Sbrissa, 1990). *A. andersoni* populations fluctuate over the growing season showing irregular patterns and decline to low levels in hot and dry summers. Phytophagous mites can build large populations in these conditions especially if predatory mites occur at low densities. Observations made in vineyards during hot summers (e.g. 2003 and 2006) showed that *K. aberrans* persisted longer than other predatory mites. This finding and encouraging results obtained in Trentino suggested the release of *K. aberrans* in apple orchards of the Veneto region inhabited by *A. andersoni* and managed by different cropping methods.

The *K. aberrans* strain considered in our study was transferred from a vineyard to apple orchards with contrasting results. Releases obtained positive results in organic orchards, although with significant variations, whereas they were not successful in conventional orchards. Pesticide use can be a key factor in explaining these results. The use of pyrethrins and spinosad were associated with low predatory mite numbers in organic orchards but did not prevent the establishment of *K. aberrans* in orchards located at Spresiano. In the remaining organic orchards the success of releases was less clear probably because of the early use of spinosad. Monteiro *et al.*, (in press) found a higher predation rate of codling moth



eggs in organic compared to conventional apple orchards. However, some organic orchards showed lower predation rates than heavily treated conventional ones. The authors discussed these differences suggesting a negative effect of spinosad on predators. We found spinosad to be harmful to *K. aberrans* in laboratory and field studies (Duso *et al.*, submitted) and this detrimental effect is likely associated to predatory mite decreases observed in mid-summer at Spresiano in the three experimental years. Predatory mite releases in conventional orchards were followed by the use of various fungicides (e.g. mancozeb and pyrimethanil) and insecticides (e.g. thiacloprid, tau-fluvalinate and emamectin) poorly selective towards predatory mites (e.g. Pozzebon *et al.*, 2002; Bernard *et al.*, 2010; Kim *et al.*, 2005). This strain proved to be resistant to chlorpyrifos (Tirello *et al.*, 2012) and female survival was slightly affected by several insecticides (e.g. neonicotinoids) in laboratory trials. However, females showed a significant decrease in fecundity when exposed to these pesticides (Duso *et al.*, submitted). We can suggest that the impact of non-selective pesticides was probably even higher on predatory mites stressed by release procedures (grapevine shoots were detached and transferred) and climatic conditions (temperatures were relatively high in June 2010 and 2011).

Factors potentially affecting failures in *K. aberrans* releases include predatory mite-host plant relationships. The best performance in *K. aberrans* releases was obtained in Florina orchard located at Spresiano. This cultivar has leaf morphology (relatively high trichome density on the leaf blade and domatia) favourable to predatory mite colonization as shown for *T. pyri* (Duso *et al.*, 2003). In previous work *K. aberrans* proved to build larger populations on cultivars having pubescent leaf morphology such as Reinette du Canada compared to other cultivars (Duso *et al.*, 2009). It has been argued that leaves having domatia or high pubescence favour the colonization of phytoseiids because of improved micro-environmental conditions (Grostal and O'Dowd, 1994), increased protection from predators (Roda *et al.*, 2000; Norton *et al.*, 2001), and retention of alternative foods (e.g. pollen) (Kreiter *et al.*, 2002; Roda *et al.*, 2001, 2003). Egg-hatching of *K. aberrans* is strongly affected by low humidity (Schausberger, 1998a) and this can be related to the association of *K. aberrans* with plant species having pubescent leaves in both natural conditions (Kreiter *et al.*, 2002) and agroecosystems (Duso, 1992b). Finally, the impact of non-selective pesticides can be lower on apple cultivars having pubescent leaf under-surfaces (Blommers and Overmeer, 1986). Unfortunately, Florina and Golden Delicious were not inter-planted and so this hypothesis could not be tested. On the other hand our data confirm trends reported in Trentino where Golden Delicious was less favourable to *K. aberrans* colonization than other cultivars (Duso

*et al.*, 2009). *Amblyseius andersoni* did not show clear preferences for definite leaf morphology and only a slight preference for Florina when compared with other apple cultivars (Duso and Pasini, 2003). Releases of *K. aberrans* in Golden Delicious commercial orchards located in Trentino obtained better results than in the current study probably because the pesticides used were less detrimental and their use was delayed with respect to the release time.

Interspecific competition is another significant factor affecting the outcome of releases (Croft, 1994; Croft and McRae, 1992). Interactions among *T. pyri*, *E. finlandicus* and *K. aberrans* on apple seedlings showed the dominance of *T. pyri* over the other two species because this predator was superior in terms of interspecific predation and survival in conditions of prey and water scarcity (Schausberger, 1997, 1998b, 1999). Comparative studies on interspecific predation showed that *A. andersoni* females were more aggressive than *K. aberrans* females towards heterospecific larvae (Schausberger and Croft, 2000). The competition with the more aggressive *A. andersoni*, which were probably more resistant than *K. aberrans* to pesticides, could be another factor explaining failures in conventional orchards. However, data from Spresiano orchards highlight that *K. aberrans* can compete successfully with *A. andersoni* under a minor pesticide pressure. Recent studies show that the conversion of food in *K. aberrans* is more efficient than in *A. andersoni* regardless of prey species (Lorenzon *et al.*, 2012). This phenomenon can represent a clear advantage for predatory mite persistence in conditions of prey scarcity as observed in our study.

In conclusion, pesticides and host plant features are the main obstacles to the establishment of *K. aberrans* in apple orchards. Improvement of IPM strategies according to Directive 128/2009 will offer new chances to achieve better results in biological control programmes.

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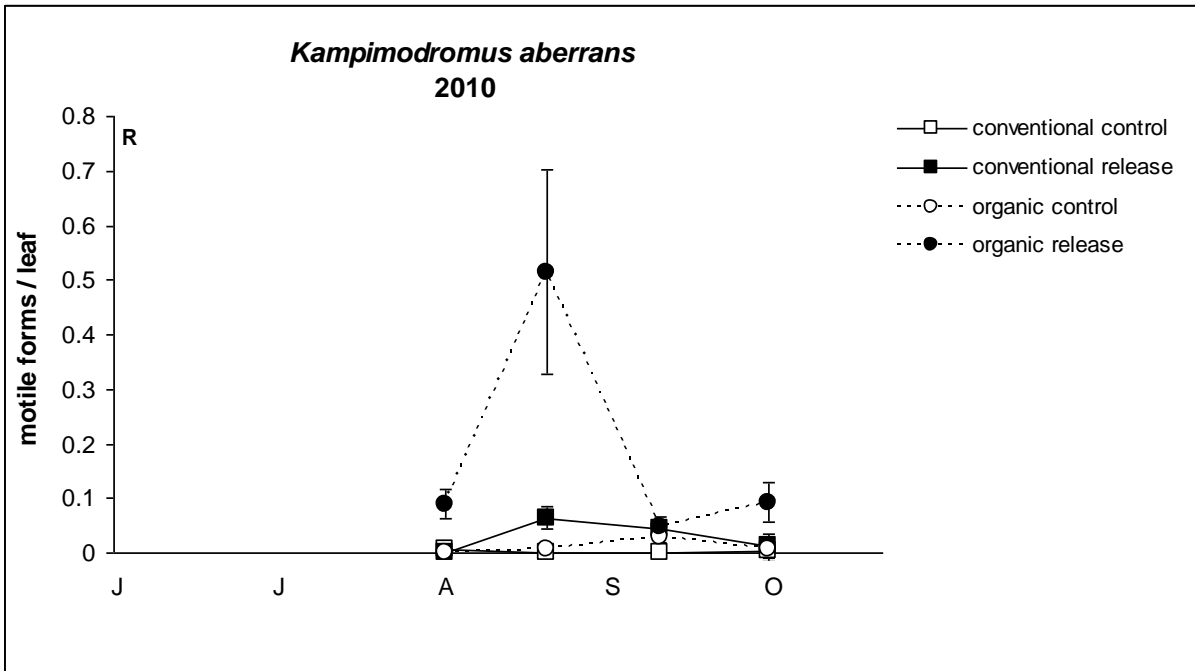


Figure 1- Seasonal abundance of *K. aberrans* in release and control plots of conventional and organic apple orchards during 2010. Releases (R) were in June.

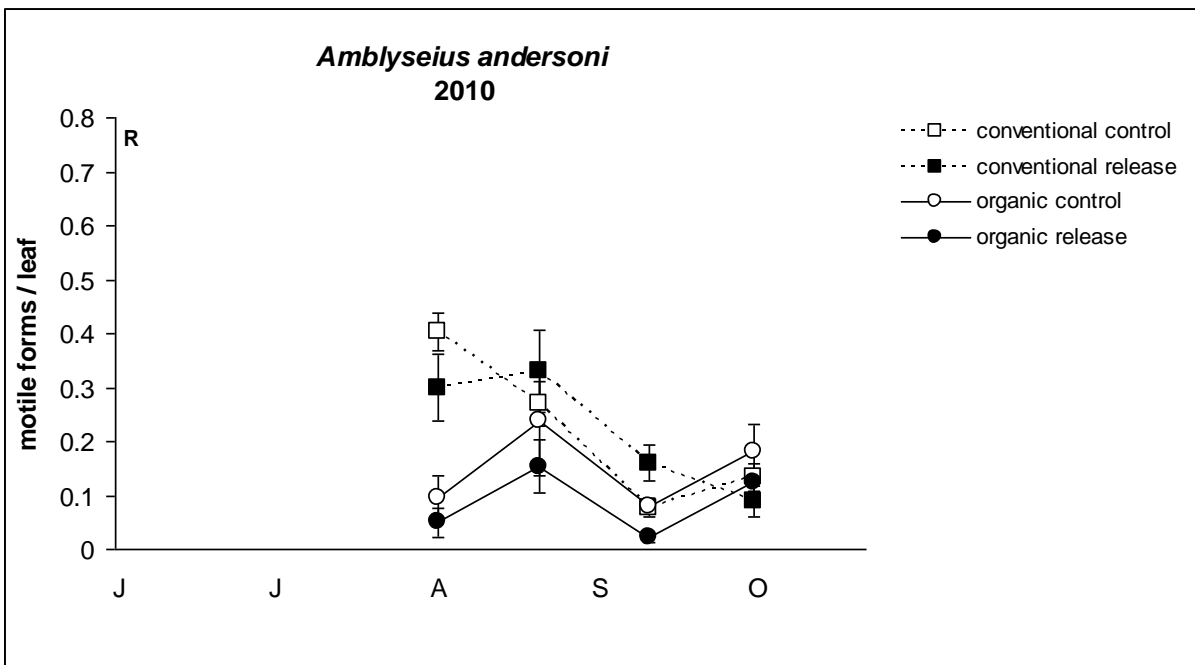


Figure 2 - Seasonal abundance of *A. andersoni* in release and control plots of conventional and organic apple orchards during 2010. Releases (R) were in June.

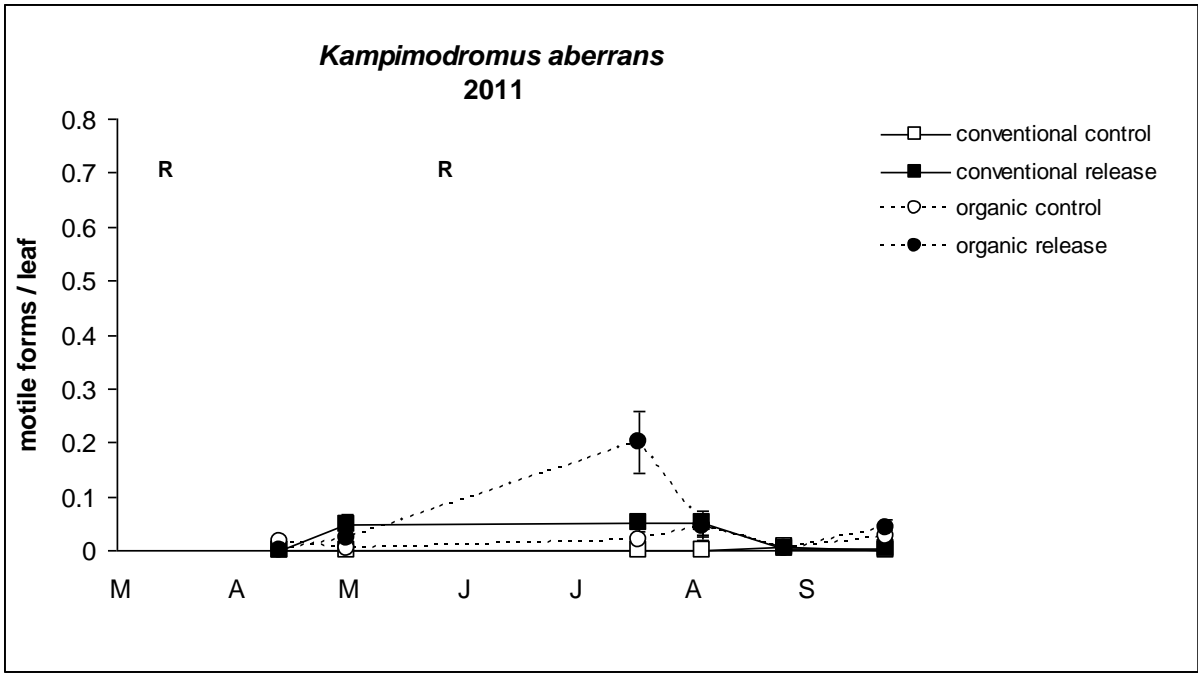


Figure 3 - Seasonal abundance of *K. aberrans* in release and control plots of conventional and organic apple orchard during 2011. Releases (R) were in February and June.

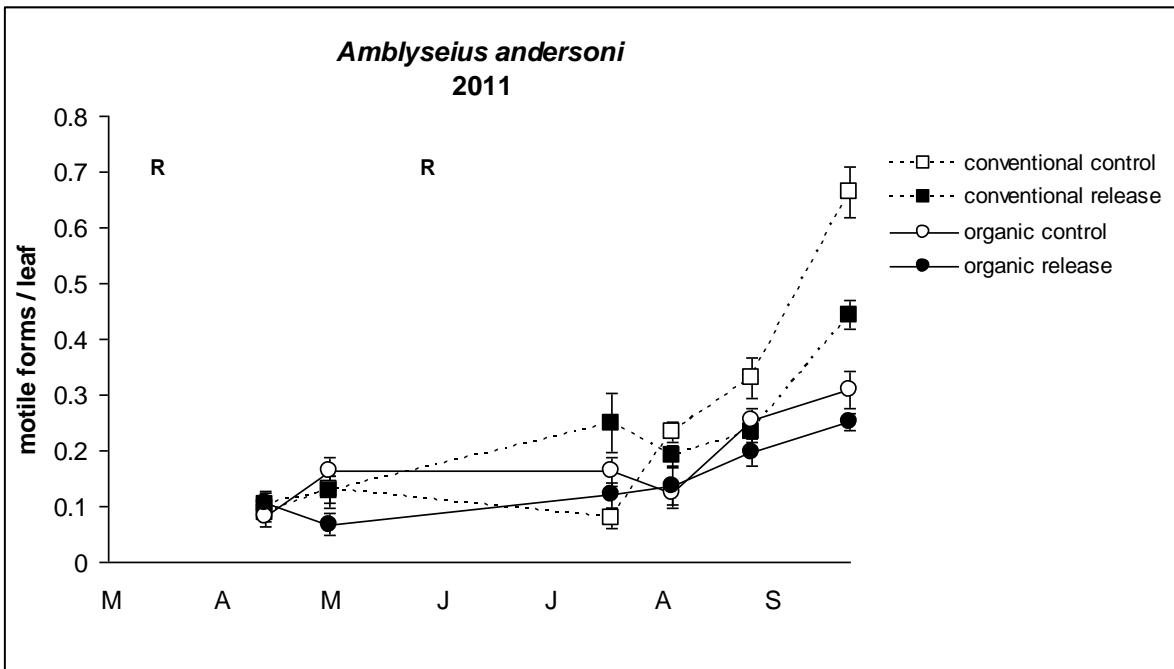


Figure 4 - Seasonal abundance of *A. andersoni* in release and control plots of conventional and organic apple orchards during 2011. Releases (R) were in February and June.

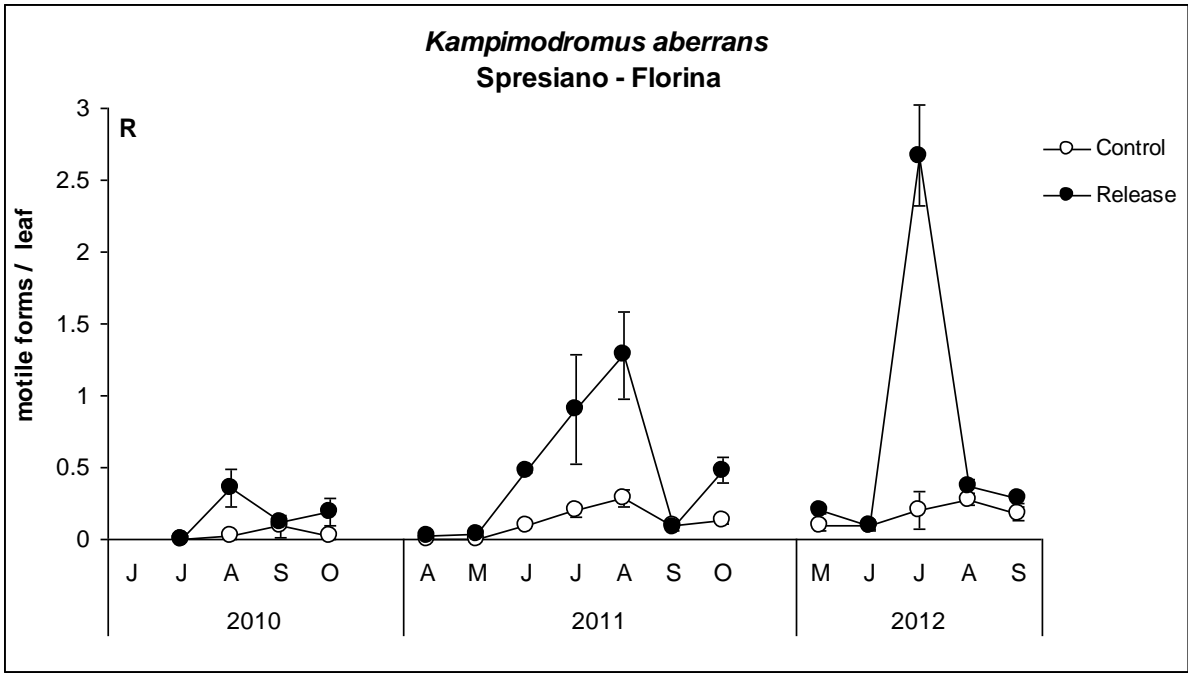


Figure 5 - Seasonal abundance of *K. aberrans* in release and control plots of Florina apple orchard at Spresiano during 2010-2012. Releases (R) were in June.

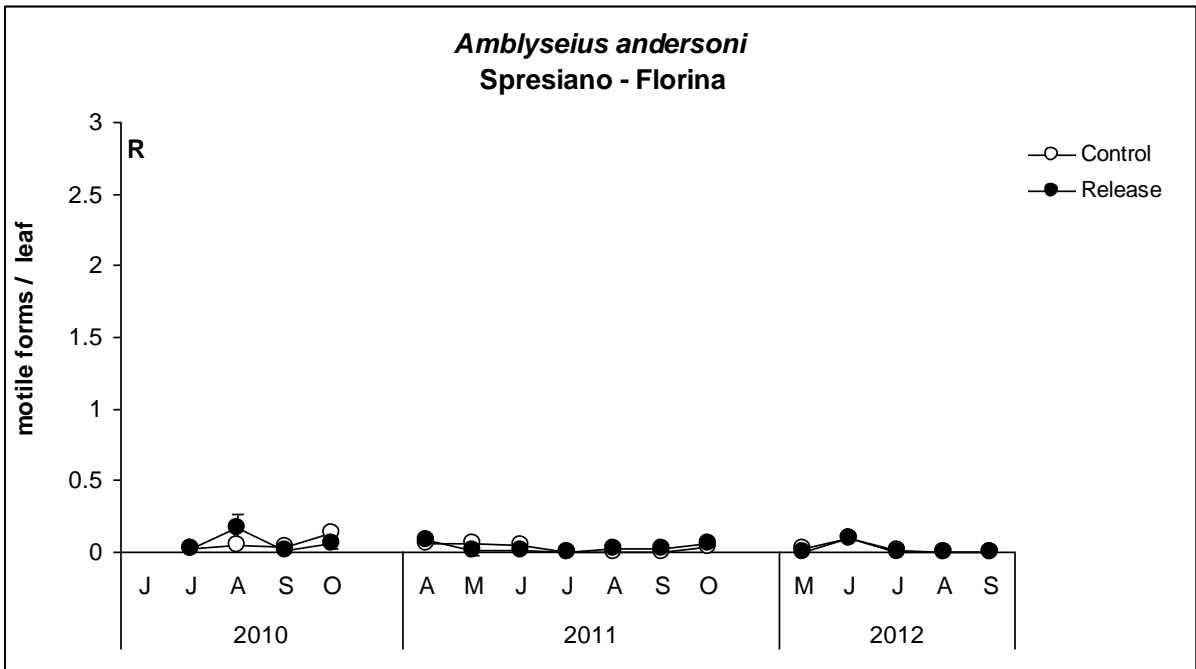


Figure 6 - Seasonal abundance of *A. andersoni* in release and control plots of Florina orchard in Spresiano during 2010-2012. Releases (R) were in June.

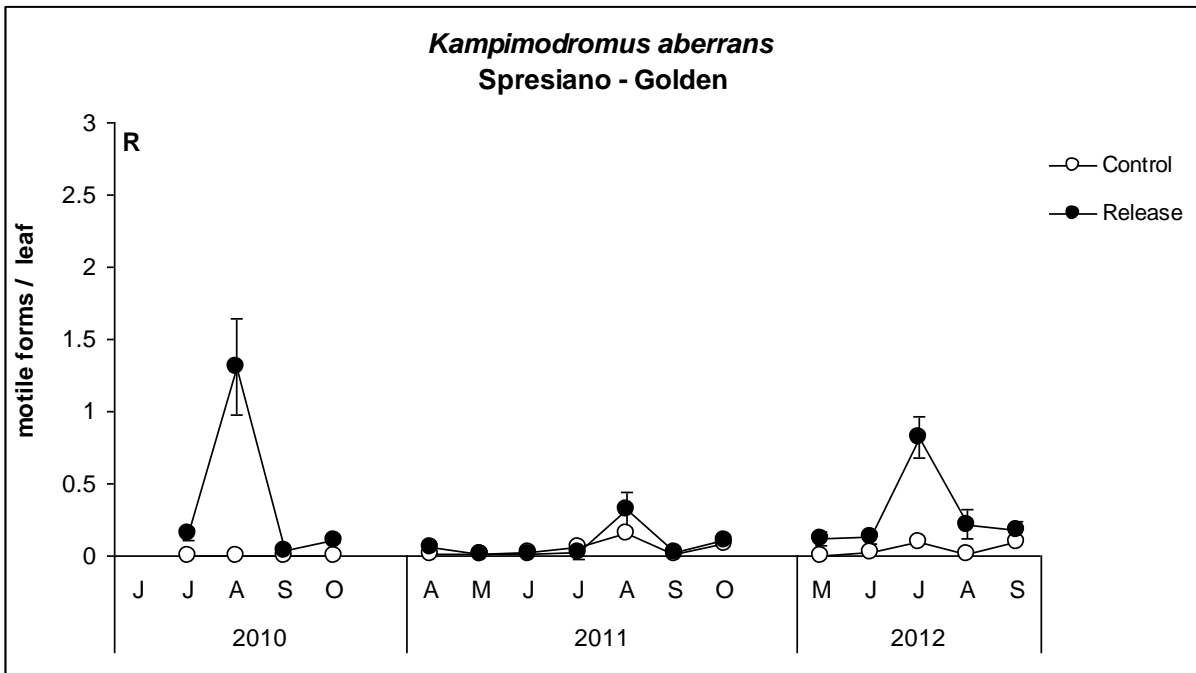


Figure 7 - Seasonal abundance of *K. aberrans* in release and control plots of Golden Delicious apple orchard at Spresiano during 2010-2012. Releases (R) were in June.

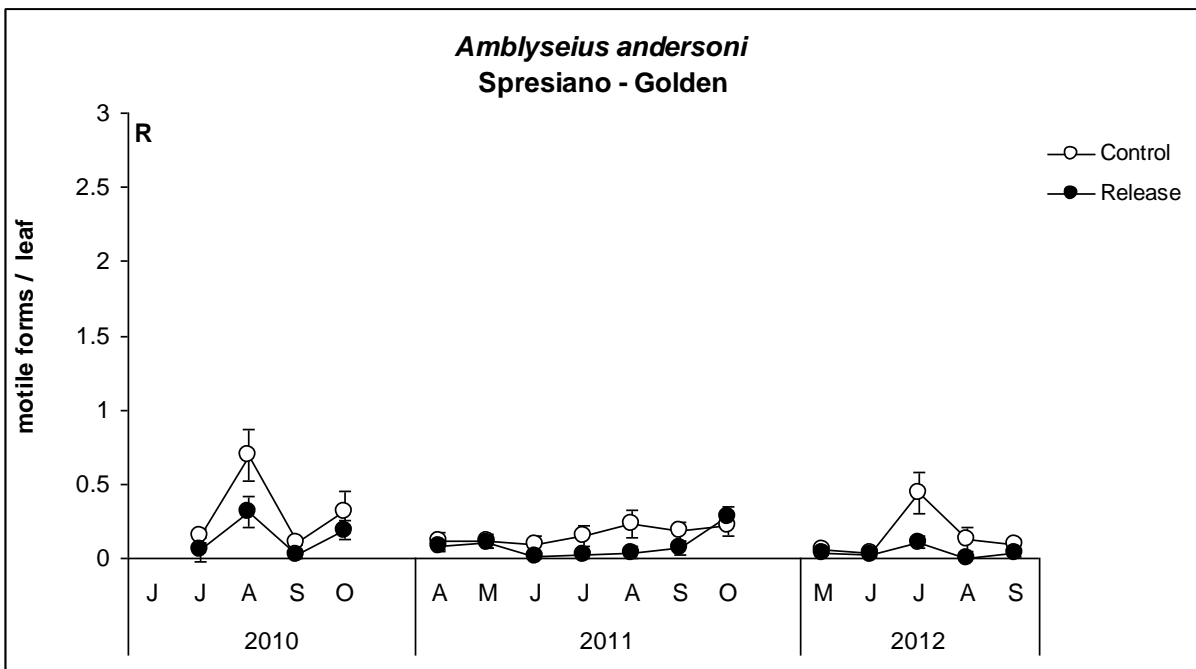


Figure 8 - Seasonal abundance of *A. andersoni* in *K. aberrans* release and control plots of Golden Delicious orchard at Spresiano during 2010-2012. Releases (R) were in June.

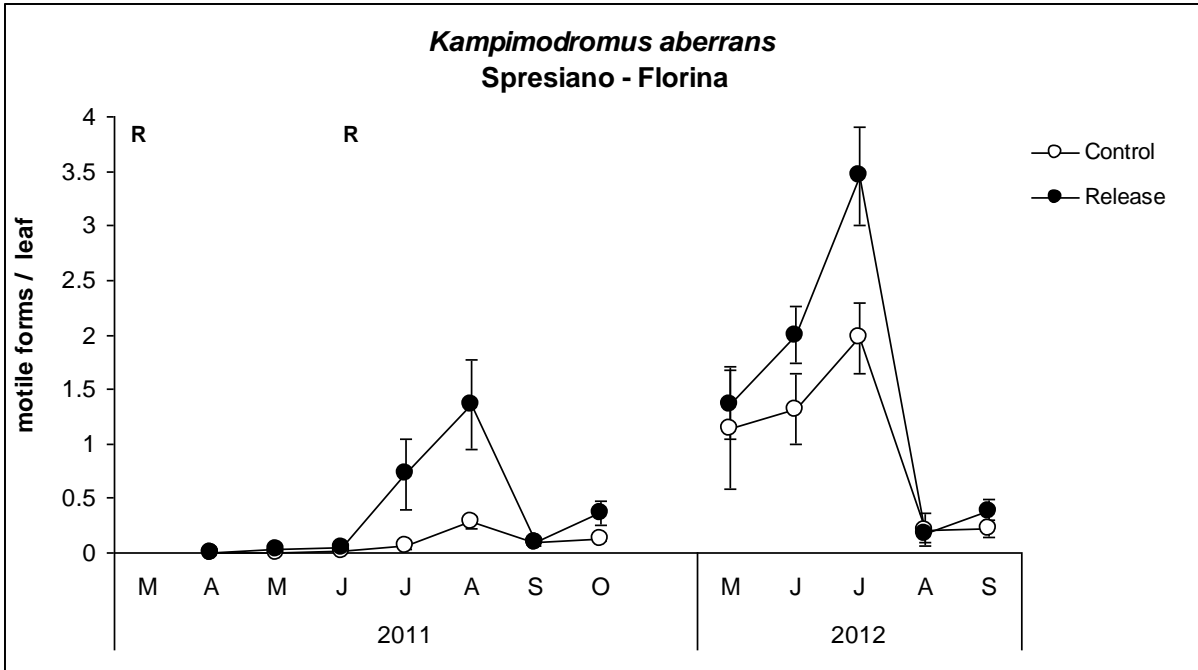


Figure 9 - Seasonal abundance of *K. aberrans* in release and control plots in Florina orchard at Spresiano during 2011-2012. Releases (R) were in February and June 2011.

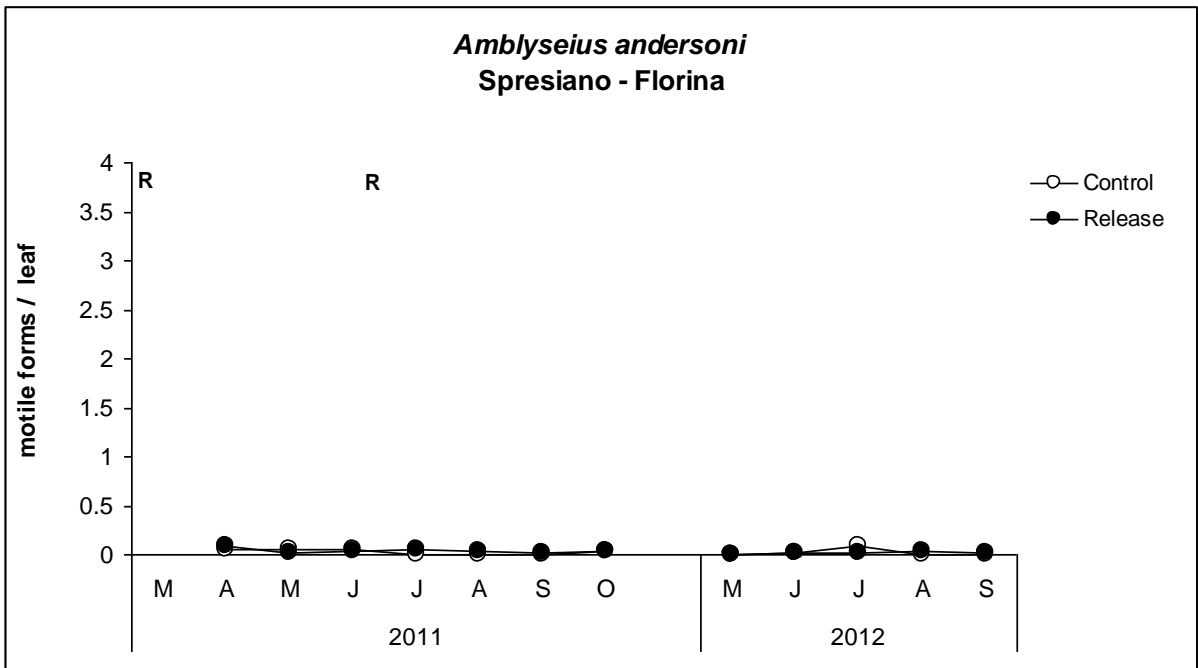


Figure 10 - Seasonal abundance of *A. andersoni* in release and control plots of Florina orchard in Spresiano during 2011-2012. Releases (R) were in February and June 2011.

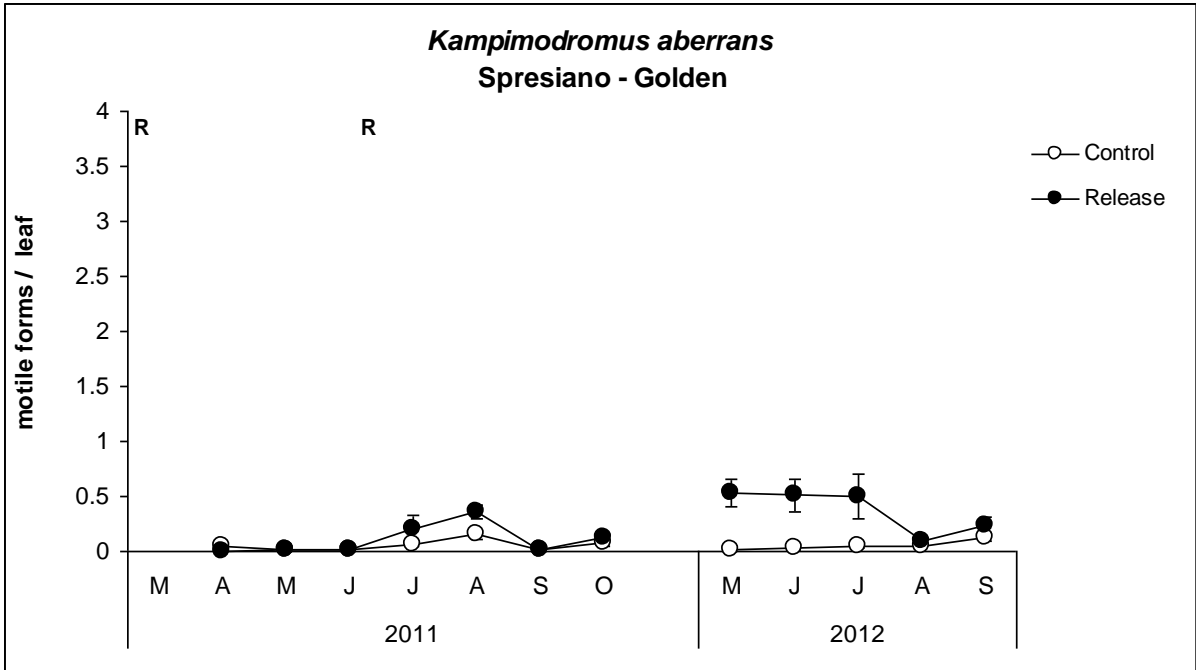


Figure 11 - Seasonal abundance of *K. aberrans* in release and control plots of Golden Delicious orchard in Spresiano during 2011-2012. Releases were in February and June 2011.

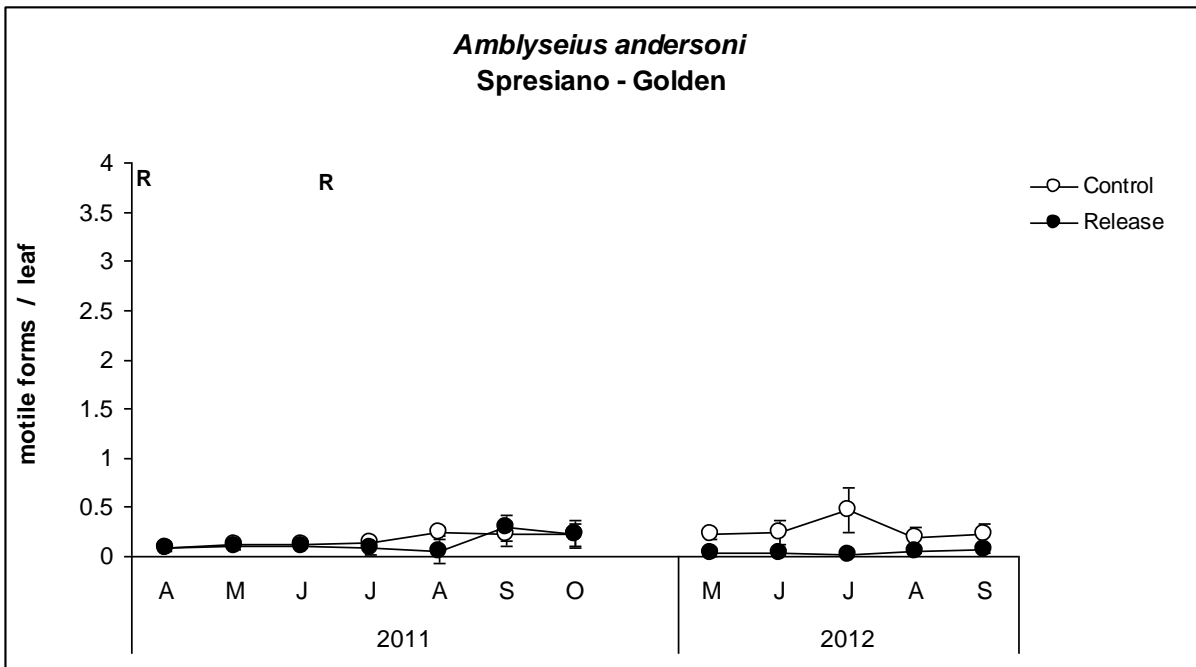


Figure 12 - Seasonal abundance of *A. andersoni* in release and control plots of Golden Delicious orchard in Spresiano during 2011-2012. Releases (R) were in February and June 2011.

**Table 1** - Locations of experimental orchards, management type, cultivar, and insecticides used from 2010 to 2012.

<b>Orchard</b>	<b>Type</b>	<b>Cultivar</b>	<b>Year</b>	<b>Active Ingredient (date)</b>
Spresiano	Organic	Florina	2010	Pyrethrins (7 May, 20 July), Spinosad (28 July, 18 and 28 August)
			2011	Pyrethrins (5 April, 7 and 13 May, 6 July), Spinosad (16 and 28 July, 18 August)
			2012	Pyrethrins (28 June, 8 July), Spinosad (4, 19 and 27 July)
Spresiano	Organic	Golden Delicious	2010	Pyrethrins (7 May, 20 July), Spinosad (28 July, 18 and 28 August)
			2011	Pyrethrins (5 April, 7 and 13 May, 6 July), Spinosad (16 and 28 July, 18 August)
			2012	Pyrethrins (28 June, 8 July), Spinosad (4, 19, 27 July)
Pernumia	Organic	Golden Delicious	2010	Spinosad (28 July, 6 and 27 August)
			2011	Spinosad (24 May, 16 and 23 July)
San Pietro Viminario	Organic	Golden Delicious	2011	Pyrethrins (4 April), Spinosad (7 May, 15 July)
Lancenigo	Conventional	Golden Delicious	2010	Thiacloprid (8 June), Chlorpyrifos (28 June), Methoxyfenozide (31 July)
			2011	Thiacloprid (13 June), Chlorpyrifos (5 July), Methoxyfenozide (2 August)
Povegliano 1	Conventional	Golden Delicious	2010	Emamectin (15 July)
			2011	Tau-fluvalinate (20 May), Chlorpyrifos (20 May), Emamectin (13 July), Methoxyfenozide (25 August)
Povegliano 2	Conventional	Golden Delicious	2010	Thiacloprid (7 June), Chlorpyrifos (26 June, 9 July), Methoxyfenozide (31 July), Emamectin (10 Sep)
			2011	Thiacloprid (30 June, 12 July), Emamectin (10 August)

## Chapter III

### **Predation on heterospecific larvae by adult females of *Kampimodromus aberrans*, *Amblyseius andersoni*, *Typhlodromus pyri* and *Phytoseius finitimus* (Acari: Phytoseiidae)**

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Manuscript in preparation as:

Shakeel Ahmad, Alberto Pozzebon, Carlo Duso - Predation on heterospecific larvae by adult females of *Kampimodromus aberrans*, *Amblyseius andersoni*, *Typhlodromus pyri* and *Phytoseius finitimus* (Acari: Phytoseiidae)

I collected most of the data, contributed to statistical analysis and drafted a manuscript



## Abstract

The longevity, prey consumption, fecundity and prey conversion rates of *Kampimodromus aberrans* (Oudemans), *Amblyseius andersoni* (Chant), *Typhlodromus pyri* Scheuten and *Phytoseius finitimus* Ribaga females fed with heterospecific larvae were assessed in the laboratory. Moreover, the survival curves and age-specific oviposition of predatory mites fed on pollen were compared with those obtained on heterospecific larvae. Results suggest that *A. andersoni* should be considered as the intraguild predator. At the same time *A. andersoni* appeared to be the least efficient in food conversion. *P. finitimus* appears to suffer from intraguild predation, and its efficiency in food conversion is not superior to that of *K. aberrans* and *T. pyri*. Differences in the profiles of *K. aberrans* and *T. pyri* are less clear. The comparison between pollen and prey diets confirmed the positive effect of pollen on the fecundity of all four predatory mite species. Fecundity was higher on pollen than on predatory mite larvae. Implications in interspecific competition in a low prey availability scenario are discussed.

## Introduction

Species sharing the same habitats and resources may have to compete when the latter are limited. Competition can be distinguished as either direct (interference) or indirect competition (exploitation); interspecific predation belongs to the former type and can produce significant effects on competitors living in a community (Begon *et al.*, 1986). Interspecific competition and predation have important consequences on the structure and population dynamics of ecological communities (Connell, 1983; Schoener, 1983; Sih *et al.*, 1985). A more complex interaction that combines competition and predation is the intraguild predation that occurs when two species competing for the same limited resource can also prey on each other (Polis *et al.*, 1989). Predatory mites of the Phytoseiidae family can coexist and compete for prey in the form of phytophagous mites. Some phytoseiid mites prey also on phytoseiid competitors, especially when the mite prey density is low (e.g., Yao and Chant, 1989; Croft and Croft, 1993; Zhang and Croft, 1995).

The predatory mites *Kampimodromus aberrans* (Oudemans), *Amblyseius andersoni* (Chant), *Typhlodromus pyri* Scheuten and *Phytoseius finitimus* Ribaga are all generalist predatory mites (McMurtry and Croft, 1997). They are commonly found in orchards and vineyards in Europe and elsewhere (e.g., Chant, 1959; El Borolossy and Fischer-Colbrie, 1989; Nicotina and Cioffi, 2001; Kreiter *et al.*, 2000; Duso *et al.*, 2012). These species play an important role in the control of tetranychids and eriophyoids (Acari Tetranychidae,

Eriophyidae), and may be dominant within predatory mite communities (Collyer, 1964; Ivancich Gambaro, 1975; McMurtry, 1982; Duso, 1989; Blommers, 1994; Solomon *et al.*, 2000; McMurtry and Croft, 1997; Papaioannou-Souliotis *et al.*, 1999). They can share the same habitats and have overlapping food ranges (e.g., Overmeer, 1981; Dicke *et al.*, 1990; Schausberger, 1991; Duso and Camporese, 1991; Lorenzon *et al.*, 2012; Duso *et al.*, 2012) and likely interact with each other through predation.

Field studies provided some trends in interspecific competition among generalist predatory mites. Single and mixed releases of *K. aberrans*, *T. pyri* and *A. andersoni* in north-Italian vineyards were done to identify optimal biocontrol strategies for controlling phytophagous mites (Duso, 1989; Duso *et al.*, 1991; Duso and Pasqualetto, 1993; Camporese and Duso, 1996; Duso and Vettorazzo, 1999). Field experiments in apple orchards suggested the importance of interspecific competition in predator-prey communities (Croft and Mac Rae, 1992a, 1992b; Croft *et al.*, 1992; Croft, 1994). Direct competition might be the reason for the dominance of one species. However in the field, the differential impact of pesticides due to the possible presence of resistant strains can be a confounding factor. Laboratory studies thus clarified the significance of intraspecific and interspecific competition in affecting the dominance of a particular intraguild species (Croft and Croft, 1993, 1996; Croft *et al.*, 1996).

Predation on con or heterospecific life stages is common among predatory mites, and this can influence the relative dominance of one species over another (Helle and Sabelis, 1985; Schausberger and Croft, 2000). Various aspects of cannibalism and interspecific predation in both adult and immature phytoseiid mites have been considered in previous investigations (e.g., Yao and Chant, 1989; MacRae and Croft, 1993, 1997; Croft *et al.*, 1996, 1998; Monetti and Croft, 1997; Schausberger, 2003). The propensity to cannibalism and intraguild predation appear to be related to diet specialization (Schausberger and Croft, 1999, 2000). More advantage can be gained by exploiting phytoseiid intraguild prey than by cannibalism. The tendency is often the opposite for specialist predatory mites (Croft *et al.*, 1996; Schausberger, 1999; Schausberger and Croft, 2000a, 200b). Some generalist predatory mites can discriminate between con- and hetero-specific juveniles as prey (Schausberger, 1997, 1999). Feeding on hetero-specifics or con-specifics can affect juvenile survival, development and reproduction of predatory mites (Schausberger, 1997; Schausberger and Croft, 2000). Interspecific competition can influence their population growth and persistence on plants when prey is scarce (Schausberger, 1998). The effects of single or mixed population releases of *Euseius finlandicus* (Oudemans), *T. pyri* and *K. aberrans* were studied on apple seedlings infested by *Panonychus ulmi* (Koch) (Schausberger, 1998). The performance of predatory

mites was compared in conditions of diminishing prey availability. In single releases, each predatory species persisted to the end of the experiment despite prey scarcity, but *T. pyri* reached higher population levels than *E. finlandicus* or *K. aberrans*. *T. pyri* was superior in competition probably because it was able a) to survive for some time without food, b) to complete juvenile development and reproduce on phytoseiid prey, and c) to forage more efficiently on spider mites when present at low densities (Schausberger, 1997, 1998).

In the absence of prey, non-prey foods are also important for the persistence of polyphagous predatory mites on plants (McMurtry and Croft, 1997). They can exploit food sources such as honeydew, plant-based substances and fungi (e.g. Gnanvossou *et al.*, 2005; Nomikou *et al.*, 2003; van Rijn and Tanigoshi, 1999; Pozzebon and Duso, 2008). Among alternative foods the importance of pollen has been extensively proved (e.g., Overmeer, 1985; McMurtry and Rodriguez, 1987; McMurtry *et al.*, 1991; Duso and Camporese, 1991). The abundance of phytoseiids on plants can be correlated to pollen availability (McMurtry and Johnson, 1965; Kennett *et al.*, 1979; Engel and Ohnesorge, 1994; Duso *et al.*, 1997; Addison *et al.*, 2000; Duso *et al.*, 2004). Some predatory mites can express higher reproductive fitness by feeding on pollen than preying on phytophagous mites (e.g., McMurtry and Johnson, 1965; Lorenzon *et al.*, 2012).

Efficiency in the utilization of food resources to produce offspring plays a central role in determining the outcome of intraguild interactions (e.g. Holt and Polis, 1997; Rosenheim *et al.*, 1995; Diehl and Feissel, 2000, Mylius *et al.*, 2001). Moreover, the presence of non-prey food sources can have implications on these interactions (Briggs and Borer, 2005; Holt and Huxel, 2007), and efficiency in their exploitation is a key aspect (Heithaus, 2001; Daugherty *et al.*, 2007). Life history parameters of predatory mites can represent food source exploitation efficacy, and their assessment can help in understanding the role of food sources on interspecific interactions (e.g., Duso and Camporese, 1991; Schausberger, 1992; Tanigoshi *et al.*, 1993; van Rijn and Tanigoshi, 1999; Onzo *et al.*, 2005).

Investigations on interspecific predation among *K. aberrans*, *A. andersoni*, *T. pyri* did not consider all predator-predator interactions and *P. finitimus* has not been included within this framework. The primary objective of the present study was to fill this gap. The longevity, prey consumption and fecundity rates of predatory mite females fed with larvae of heterospecifics were assessed in the laboratory. We also estimated the conversion rate of prey into egg biomass for the four predatory mites. Secondly, due to the importance of pollen for persistence of these predatory mites in the absence of prey, the survival curves and age-

specific oviposition of predatory mites fed on pollen were compared with those obtained on heterospecific prey.

## **Materials and Methods**

### **Stock cultures**

*Kampimodromus aberrans*, *A. andersoni*, *P. finitimus* and *T. pyri* strains were collected from apple orchards and vineyards in North-East Italy where they were dominant among predatory mites. Stock cultures were maintained in the laboratory at the Department DAFNAE, University of Padua, Legnaro (Italy). Colonies of the first three species were reared on grape leaves in controlled conditions ( $24 \pm 1$  °C,  $70 \pm 10\%$  R.H., 16 L: 8 D photoperiod regime) for at least three generations prior to the experiments. Single leaves were placed with the upper side down on pieces of foam plastic (3-4 cm thick) saturated with water and put in an open plastic tray. Wet tissue paper (hanging down in the water) was folded along its periphery for drinking water supply and to prevent mites escaping. The mites were fed with pollen of *Typha latifolia* stored at  $-20$  °C in a freezer and this was replenished every two days. A piece of transparent plastic sheet (1-2 cm<sup>2</sup>) folded in the shape of a tent, was placed over each arena as shelter and oviposition site for the mites. *T. pyri* colonies were maintained on artificial arenas (plastic tile of 8 cm x 15 cm x 0.4 cm) placed on water-saturated foam plastic (wet tissue paper serving as a barrier) in a plastic tray (Overmeer, 1981) in the same laboratory conditions as above. Mites were fed with *T. latifolia* pollen. Pieces of cotton fibers were placed on all the substrates, to serve as egg-laying sites and shelter.

### **Experimental procedures**

Longevity, survival, fecundity and prey consumption of predatory mite females fed with larvae of heterospecifics were assessed. For each predator species, cohorts of newly laid eggs were obtained by placing approximately 50 females from the laboratory colonies onto new apple leaves and allowing them to lay eggs for 24 hours. Mites were fed with *T. latifolia* pollen. Eggs were collected every 24 h and then transferred to separate rearing units consisting of an apple leaf section (5 x 5 cm) placed bottom-side down on a wet layer of cotton within a plastic box. Egg arenas were checked for larvae, nymphs and adults every 24h.

Each experiment started by placing single gravid females of the same age onto leaf arenas consisting of a section of apple leaf (2 x 2 cm) placed bottom-side down on a wet layer of cotton within a plastic box. The experimental units were maintained in climatic chambers at the above-mentioned controlled conditions. After 24 h starvation each female was offered

eight larvae of heterospecifics. The prey larvae were transferred with a camel hair brush into the arenas at 24 h intervals. In another set of arenas, gravid females were offered *T. latifolia* pollen.

There were 10-15 replicates (females) in each experimental unit with an arrangement of leaf sections with one adult predator female with eight heterospecific larvae as prey. The shriveled corpses of the dead larvae were taken as evidence of predation. Dead larvae which were only deflated were assessed to have died of starvation or other causes. Uneaten larvae were removed. If a female phytoseiid died before the trial period expired, it was discounted and another replicate conducted. The number of larvae consumed, and of eggs laid by predatory mites was recorded daily. Eggs were removed from the experimental units.

### Data analysis

In a first set of analyses we considered the performances of different predatory mites feeding on heterospecific larvae. We evaluated the effect of different prey species on female longevity (days), fecundity rate (number of eggs per female per day), prey consumption rate (prey per female per day), and prey conversion rate of prey into egg biomass (number of prey/number of eggs) of four predator species with one-way ANOVA using the GLM procedure of SAS (SAS Institute Inc., 1999) followed by multiple comparison using Tukey-Kramer tests ( $P = 0.05$ ). With the same statistical approach we compared longevity, fecundity, prey consumption and food conversion rates of different predatory mites species feeding on the same heterospecific larvae. Prior to all analyses data were checked for the respect of assumption of ANOVA and data on fecundity, prey consumption and food conversion rates were transformed in  $\log(x+1)$ , while untransformed longevity data were used.

In a second set of analyses we compared survival and age-specific oviposition curves of predatory mites feeding on prey with those feeding on pollen. Survival curves were estimated using the Kaplan–Meier method and were compared by Wilcoxon  $\chi^2$  test ( $P = 0.05$ ) using the LIFETEST procedure of SAS (Allison 1995). In this analysis we also estimated survivals of 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentiles and their 95% confidence limits. Age-specific fecundity was fitted using the NLIN procedure of SAS to the following equation based on the two-parameter Weibull density distribution function:

$$Asf_t = Eggs_{mx}abt^{b-1}e^{-atb}$$

where  $Asf_t$  is the age-specific oviposition rate observed at time  $t$ ,  $Eggs_{mx}$  is the total fecundity of a female and  $a$  and  $b$  are scale and shape parameters, respectively. In this analysis 95% confidence intervals of scale and shape parameters were also estimated. Significant differences in shape and scale parameters were established by non-overlapping 95%

confidence intervals. Pseudo- $R^2$  values were calculated according to Schabenberger (1998). Total fecundity of females feeding on pollen and prey were compared using one-way ANOVA with the GLM procedure of SAS (SAS Institute Inc., 1999) followed by multiple comparison using Tukey-Kramer tests ( $P = 0.05$ ). Data were checked for the respect of assumption of ANOVA and were transformed in  $\log(x+1)$  prior to the analysis.

## Results

### Predatory mite performance on heterospecific larvae

#### *Kampimodromus aberrans*

Longevity of *K. aberrans* females differed significantly among diets ( $F_{2, 31} = 5.46$ ;  $P = 0.009$ ). Adult females survived longer when fed with *A. andersoni* as compared to *T. pyri* larvae (Figure 1). A diet based on *P. finitimus* larvae gave intermediate results (Figure 1). Different foods showed a significant effect on *K. aberrans* fecundity rate ( $F_{2, 31} = 7.29$ ;  $P = 0.003$ ). *K. aberrans* laid more eggs when fed with *P. finitimus* than with *A. andersoni* and *T. pyri* larvae (Figure 2). Prey consumption rates of *K. aberrans* on the three prey species resulted as different ( $F_{2, 31} = 57.22$ ;  $P < 0.0001$ ). *K. aberrans* preyed more on *P. finitimus* larvae as compared to *A. andersoni* and *T. pyri* (Figure 3). Regarding prey conversion into egg biomass, results showed no significant differences for *K. aberrans* females fed with different prey species ( $F_{2, 31} = 0.18$ ;  $P = 0.834$ , Figure 4).

#### *Amblyseius andersoni*

Longevity of *A. andersoni* females was influenced by food ( $F_{2, 32} = 24.09$ ;  $P < 0.0001$ ). *A. andersoni* could survive longer when fed with *K. aberrans* larvae as compared to other prey. Longevity was higher on *P. finitimus* compared to *T. pyri* larvae (Figure 1). There were significant effects of diet on *A. andersoni* fecundity ( $F_{2, 32} = 15.23$ ;  $P < 0.0001$ ). *A. andersoni* showed significantly higher fecundity when fed with *P. finitimus* or *T. pyri* compared to *K. aberrans* larvae (Figure 2). Prey consumption rate was also influenced by prey species ( $F_{2, 32} = 55.30$ ;  $P < 0.0001$ ). *A. andersoni* females were able to consume more *P. finitimus* and *T. pyri* larvae as compared to *K. aberrans* (Figure 3). Food conversion into egg biomass resulted as different among prey species ( $F_{2, 32} = 4.62$ ;  $P = 0.017$ ). *A. andersoni* required more *K. aberrans* than *T. pyri* larvae to produce one egg while *P. finitimus* was associated to intermediate values (Figure 4).

### ***Typhlodromus pyri***

*Typhlodromus pyri* longevity was not influenced by diet ( $F_{2, 27} = 3.25$ ;  $P = 0.054$ ; Figure 1). Fecundity rate was influenced by prey ( $F_{2, 27} = 23.78$ ;  $P < 0.0001$ ) and was lowest on *A. andersoni* (Figure 2). There were no differences in the performance of *T. pyri* females fed with *P. finitimus* or *K. aberrans* larvae (Figure 2). Prey consumption of *T. pyri* was affected by prey mite species ( $F_{2, 27} = 65.39$ ;  $P < 0.0001$ ) resulting as higher on *P. finitimus* compared to *K. aberrans* and *A. andersoni* larvae (Figure 3). Moreover, *T. pyri* females consumed more *K. aberrans* than *A. andersoni* larvae (Figure 3). Conversion of prey into egg biomass by *T. pyri* females was similar on the three prey species ( $F_{2, 27} = 0.13$ ;  $P = 0.875$ ) (Figure 4).

### ***Phytoseius finitimus***

There were significant differences in the longevity of *P. finitimus* fed with heterospecific larvae ( $F_{2, 41} = 3.74$ ;  $P = 0.032$ ; Figure 1). Longevity was higher on *K. aberrans* than on *A. andersoni* (Figure 1) while values were intermediate on *T. pyri* (Table 1). Fecundity was not influenced by diet ( $F_{2, 36} = 0.08$ ;  $P = 0.921$ ; Figure 2). There were significant effects of prey species on prey consumption by *P. finitimus* females ( $F_{2, 41} = 16.15$ ;  $P < 0.0001$ ). They consumed more *K. aberrans* and *T. pyri* than *A. andersoni* larvae (Figure 3). The food conversion rate was significantly different among prey species ( $F_{2, 36} = 5.34$ ;  $P = 0.009$ ). Lowest values were calculated for *A. andersoni* compared to *K. aberrans* while *T. pyri* did not differ from the others (Figure 4).

## **Comparative performance of predatory mites on the same prey**

### ***K. aberrans*, *T. pyri* and *P. finitimus* feeding on *A. andersoni* larvae**

There were significant differences among the longevities of *K. aberrans*, *T. pyri* and *P. finitimus* fed with *A. andersoni* larvae ( $F_{2, 36} = 3.80$ ;  $P = 0.033$ ). Longevity was higher for *K. aberrans* than for *P. finitimus* while that of *T. pyri* was similar to the others (Table 1). Fecundity rate of predatory mites fed with *A. andersoni* larvae was also different ( $F_{2, 36} = 18.81$ ;  $P < 0.0001$ ), being higher for *K. aberrans* and *T. pyri* than for *P. finitimus* (Table 1). Prey consumption followed a similar trend ( $F_{2, 36} = 63.24$ ;  $P < 0.0001$ ; Table 1). In contrast, the prey conversion rate did not significantly differ among predators ( $F_{2, 32} = 0.61$ ;  $P = 0.547$ ; Table 1).

### ***K. aberrans*, *T. pyri* and *A. andersoni* feeding on *P. finitimus* larvae**

The longevity of predatory mite females feeding on *P. finitimus* larvae differed significantly ( $F_{2, 27} = 47.31$ ;  $P < 0.0001$ ). *A. andersoni* females survived longer as compared to *K. aberrans* and *T. pyri*. Fecundity rate also differed among predatory mites, resulting as higher for *T. pyri* and *K. aberrans* than for *A. andersoni* ( $F_{2, 27} = 18.50$ ;  $P < 0.0001$ ; Table 1). Prey consumption rate was significantly different among predators ( $F_{2, 27} = 15.43$ ;  $P < 0.0001$ ), resulting as higher for *A. andersoni* than for *K. aberrans* and *T. pyri* (Table 1). Prey conversion rate also differed ( $F_{2, 27} = 31.29$ ;  $P < 0.0001$ ): *A. andersoni* needed more prey than *K. aberrans* and *T. pyri* to produce one egg (Table 1).

### ***T. pyri*, *A. andersoni* and *P. finitimus* feeding on *K. aberrans* larvae**

There was a significant effect of prey on the longevity of *A. andersoni*, *T. pyri* and *P. finitimus* ( $F_{2, 36} = 7.76$ ;  $P = 0.002$ ). Longevity of *A. andersoni* females was higher than that exhibited by *T. pyri* while that of *P. finitimus* did not differ from the others (Table 1). Fecundity differed significantly among predators feeding on *K. aberrans* ( $F_{2, 36} = 29.53$ ;  $P < 0.0001$ ) and was higher for *T. pyri* compared to the other species (Table 1). Prey consumption rates on *K. aberrans* larvae differed significantly among predatory mites ( $F_{2, 36} = 90.16$ ;  $P < 0.0001$ ). Adult females of *T. pyri* and *A. andersoni* consumed more prey as compared to *P. finitimus* (Table 1). The conversion rate of food into egg biomass also differed ( $F_{2, 36} = 16.38$ ;  $P < 0.0001$ ). The most efficient was *T. pyri* and the least *A. andersoni*, while *P. finitimus* showed intermediate performance (Table 1).

### ***K. aberrans*, *A. andersoni* and *P. finitimus* feeding on *T. pyri* larvae**

Longevity of females was similar among predators ( $F_{2, 32} = 0.03$   $P = 0.969$ ) fed with *T. pyri* larvae (Table 1). In contrast, fecundity differed ( $F_{2, 31} = 9.05$ ;  $P = 0.008$ ), being higher for *A. andersoni* and *K. aberrans* than for *P. finitimus* (Table 1). Prey consumption rate was significantly different among predatory mites ( $F_{2, 32} = 117.58$ ;  $P < 0.0001$ ). *A. andersoni* showed the highest prey consumption rate, and *P. finitimus* consumed less prey than *K. aberrans* (Table 1). The prey conversion rate also differed ( $F_{2, 32} = 4.73$ ;  $P = 0.016$ ), being lower for *K. aberrans* than for *A. andersoni*, and intermediate for *P. finitimus* (Table 1).



## Survival and age-specific oviposition curves on pollen and prey

### *Kampimodromus aberrans*

Survival curves of *K. aberrans* fed with different food sources were different ( $\chi^2 = 14.49$ ;  $df = 3$ ;  $P < 0.0001$ ; Figure 5). Feeding on pollen or *T. pyri* resulted in similar curves ( $\chi^2 = 0.22$ ;  $df = 1$ ;  $P = 0.635$ ; Table 2; Figure 5). Survival was lower on these food sources than on *P. finitimus* (vs. pollen:  $\chi^2 = 9.47$ ;  $df = 1$ ;  $P = 0.002$ ; vs. *T. pyri*:  $\chi^2 = 10.19$ ;  $df = 1$ ;  $P = 0.001$ ; Table 2; Figure 5), and *A. andersoni* (vs. pollen:  $\chi^2 = 4.71$ ;  $df = 1$ ;  $P = 0.029$ ; vs. *T. pyri*:  $\chi^2 = 5.02$ ;  $df = 1$ ;  $P = 0.024$ ; Table 2; Figure 5). No differences were found between survival curves of *K. aberrans* feeding on *P. finitimus* or *A. andersoni* ( $\chi^2 = 3.14$ ;  $df = 1$ ;  $P = 0.076$ ; Table 2; Figure 5). Total fecundity of *K. aberrans* was influenced by food sources ( $F_{3, 40} = 8.24$ ;  $P < 0.001$ ; Table 3). A higher number of eggs was laid with pollen compared to prey (Figure 6; Table 3). Scale parameter of oviposition curves was higher for females fed with pollen than for those fed with *A. andersoni* (Figure 6; Table 3). No differences were observed in shape parameter (Figure 6; Table 3).

### *Amblyseius andersoni*

Food source influenced survival curves of *A. andersoni* ( $\chi^2 = 48.11$ ;  $df = 3$ ;  $P < 0.0001$ ; Figure 5). Feeding on pollen or on *T. pyri* resulted in similar survival ( $\chi^2 = 0.46$ ;  $df = 1$ ;  $P = 0.493$ ; Table 2; Figure 5). On these food sources survival was lower than on *P. finitimus* (vs. pollen:  $\chi^2 = 18.05$ ;  $df = 1$ ;  $P < 0.0001$ ; vs. *T. pyri*:  $\chi^2 = 17.98$ ;  $df = 1$ ;  $P = 0.001$ ; Table 2; Figure 5), and *K. aberrans* (vs. pollen:  $\chi^2 = 26.89$ ;  $df = 1$ ;  $P < 0.0001$ ; vs. *T. pyri*:  $\chi^2 = 26.59$ ;  $df = 1$ ;  $P < 0.0001$ ; Table 2; Figure 5). No differences were found between survival curves of *A. andersoni* fed with *P. finitimus* or *K. aberrans* ( $\chi^2 = 3.46$ ;  $df = 1$ ;  $P = 0.069$ ; Table 2; Figure 5). Fecundity of *A. andersoni* was influenced by food sources ( $F_{3, 41} = 14.88$ ;  $P < 0.0001$ ; Table 3). Higher fecundity was found in predatory mites feeding on pollen compared to prey (Figure 6; Table 3). Among prey, a higher number of eggs was laid preying on *P. finitimus* compared to *T. pyri* (Figure 6; Table 3). The fecundity of *A. andersoni* fed with *K. aberrans* reached intermediate values (Figure 6; Table 3). Scale parameter of age-specific oviposition curve on pollen was similar to *T. pyri* but higher than *K. aberrans* and *P. finitimus* (Figure 6; Table 3). Moreover, the scale parameter was higher for *T. pyri* compared to *K. aberrans* (Figure 6; Table 3). No differences were observed in shape parameter (Figure 6; Table 3).

### ***Typhlodromus pyri***

Food source influenced survival curves of *T. pyri* ( $\chi^2 = 11.47$ ;  $df = 3$ ;  $P = 0.009$ ; Figure 5). Survival curve on pollen was similar to those on *K. aberrans* ( $\chi^2 = 0.46$ ;  $df = 1$ ;  $P = 0.493$ ; Table 2; Figure 5), and *A. andersoni* ( $\chi^2 = 3.46$ ;  $df = 1$ ;  $P = 0.06$ ; Table 2; Figure 5), but lower compared to *P. finitimus* ( $\chi^2 = 6.30$ ;  $df = 1$ ;  $P = 0.012$ ; Table 2; Figure 5). No differences emerged between survival curves on *K. aberrans* and *A. andersoni* ( $\chi^2 = 0.03$ ;  $df = 1$ ;  $P = 0.856$ ; Table 2; Figure 5), while on these prey survival was lower than on *P. finitimus* (vs. *T. pyri*:  $\chi^2 = 4.97$ ;  $df = 1$ ;  $P = 0.025$ ; vs. *A. andersoni*:  $\chi^2 = 4.72$ ;  $df = 1$ ;  $P = 0.03$ ; Table 2; Figure 5). Total fecundity of *T. pyri* differed among food sources ( $F_{3, 36} = 51.57$ ;  $P < 0.0001$ ; Table 3), with higher level of fecundity observed on pollen than on prey (Table 3). Among the latter, a higher number of eggs was laid by preying on *K. aberrans* and *P. finitimus* compared to *A. andersoni* larvae (Table 3). No differences were found in parameters of age-specific oviposition curves of *T. pyri* (Figure 6; Table 3).

### ***Phytoseius finitimus***

Food sources influenced survival curves of *P. finitimus* ( $\chi^2 = 10.97$ ;  $df = 3$ ;  $P = 0.012$ ; Table 2; Figure 5). Survival curve on pollen was similar to that observed on *T. pyri* ( $\chi^2 = 3.23$ ;  $df = 3$ ;  $P = 0.072$ ; Table 2; Figure 5) and *A. andersoni* ( $\chi^2 = 5.81$ ;  $df = 3$ ;  $P = 0.054$ ; Table 2; Figure 5), but lower compared to *K. aberrans* ( $\chi^2 = 9.68$ ;  $df = 3$ ;  $P = 0.008$ ; Table 2; Figure 5). However, no differences were observed among prey (*T. pyri* vs. *K. aberrans*:  $\chi^2 = 1.28$ ;  $df = 3$ ;  $P = 0.288$ ; *T. pyri* vs. *A. andersoni*:  $\chi^2 = 2.49$ ;  $df = 3$ ;  $P = 0.114$ ; *A. andersoni* vs. *K. aberrans*:  $\chi^2 = 0.69$ ;  $df = 3$ ;  $P = 0.404$ ; Table 2; Figure 5). Oviposition curves were influenced by food sources. Total fecundity of *P. finitimus* differed among food sources ( $F_{3, 50} = 17.29$ ;  $P < 0.0001$ ; Table 3), resulting as higher on pollen compared to prey (Table 3). No differences were found in parameters of age-specific oviposition curves of *P. finitimus* (Figure 6; Table 3).

## Discussion

When prey or alternative foods are scarce, interspecific predation can be common among phytoseiids and is a key factor explaining the dominance of predatory mite species in natural and agricultural ecosystems (Yao and Chant, 1989). Predator-predator interactions in perennial crops were investigated in Oregon (USA) apple orchards inhabited by specialist and generalist predatory mites, in particular *Metaseiulus occidentalis* (Nesbitt), *Neoseiulus fallacis* (Garman), *T. pyri* and *A. andersoni* (Croft *et al.*, 1992; Croft and McRae, 1992a, 1992b; Croft, 1994); the impact of interspecific and intraspecific competition among these species was evaluated in the laboratory allowing an interpretation of field results (Zhang and Croft, 1995a, 1995b; Croft and Croft, 1996; Croft *et al.*, 1996). Investigations carried out in Austrian apple orchards and in laboratory studies highlighted the importance of interspecific competition among the polyphagous predatory mites *Euseius finlandicus* (Oudemans), *K. aberrans* and *T. pyri* (Schausberger, 1997, 1998, 1999). These and additional investigations showed that *T. pyri*, *K. aberrans* and *A. andersoni* were able to sustain survival and oviposition by intraguild predation (Schausberger, 1997, 1998, 1999; Schausberger and Croft, 2000a). *Amblyseius andersoni* proved to be more aggressive than *T. pyri*, *K. aberrans* and many other species, showing a propensity to displace competitors when coexisting (Zhang and Croft, 1995b; Croft and Croft, 1996; Schausberger and Croft, 2000b). *Typhlodromus pyri* was less aggressive than *A. andersoni* but more than specialist predatory mites (Schausberger and Croft, 2000b); moreover, it can avoid predation and survive longer without food than other predatory mite mites (Croft and Croft, 1996; Schausberger, 1997, 1999a, 1999b). The profile of *K. aberrans* was less definite: it was less aggressive and gained less advantage than *T. pyri* from predation on heterospecific immatures (Schausberger, 1999a, 1999b).

Experiments aimed at assessing the outcome of predatory mite releases in vineyards and orchards suggested that direct and indirect competition were probably involved in interactions among *K. aberrans*, *T. pyri*, *A. andersoni* and *P. finitimus* (Duso, 1989; Duso *et al.*, 1991; Duso and Pasqualetto, 1993; Duso and Vettorazzo, 1999; Duso *et al.*, 2009). Some trends that emerged in vineyards (e.g. the competition between *A. andersoni* and *T. pyri*) were partly explained by results of the above-mentioned studies. However, the dominance of *K. aberrans* that emerged in most of these field studies remained unexplained. Interactions between *A. andersoni* and *K. aberrans* have been poorly investigated in the laboratory, and *Phytoseius* species have not been considered in such studies. In the present work we reproduced interspecific competition among these four predatory mite species in controlled conditions. Predation on juveniles by predatory mite females is probably more common than interspecific

predation among adult females or immatures. Previous investigations showed that predation on heterospecific larvae is a key aspect in intraguild predation among predatory mites (e.g., Schausberger 1997, 1999a; Croft and Zhang, 1995). In the present paper we offered heterospecific prey or pollen to *K. aberrans*, *A. andersoni*, *T. pyri* and *P. finitimus* in order to compare their performance. In contrast with previous observations on interspecific predation, predatory mite females were maintained on prey larvae or pollen until their death and this can explain some discrepancies. Adult females of *K. aberrans*, *T. pyri*, *A. andersoni* and *P. finitimus* were able to survive and oviposit on heterospecific larvae. Their response to different prey offered showed some trends that can contribute to delineate their role in intraguild predation. *A. andersoni* consumed more *T. pyri* and *P. finitimus* than *K. aberrans* larvae and its fecundity was higher on the first two prey species. A diet based on *T. pyri* larvae was associated to the highest conversion rate of food into egg biomass; longevity was higher for predatory mite females fed with *K. aberrans* larvae. *K. aberrans* preyed more *P. finitimus* than *T. pyri* and *A. andersoni* larvae and its fecundity resulted as higher on the first species. The conversion of food into egg biomass did not show a precise trend, while the longevity of *K. aberrans* resulted as higher when fed with *A. andersoni*. *Typhlodromus pyri* consumed more *P. finitimus* (and to a lesser extent *K. aberrans*) larvae than *A. andersoni* and laid more eggs on the first two species. No clear trends emerged considering conversion rate and longevity. *Phytoseius finitimus* preyed more on *K. aberrans* and *T. pyri* than *A. andersoni* larvae but fecundity was not affected by prey; predatory mite females fed with *A. andersoni* larvae were more efficient in converting food into egg biomass, but those fed with *K. aberrans* larvae lived longer. These results suggest possible outcomes when species coexist and enter into intraguild predation. In terms of predation rate and fecundity, *A. andersoni* seems to be advantaged over *T. pyri*, *T. pyri* over *K. aberrans*, and *A. andersoni*, *K. aberrans* and *T. pyri* over *P. finitimus*. Most of these results agree with those reported in previous studies and some of them can be explained by the aggressiveness of predatory mites that is related to body size (Zhang and Croft, 1995b; Croft and Croft, 1996; Croft *et al.*, 1996; Schausberger, 1997, 1998, 1999; Schausberger and Croft, 2000). Prey consumption was always high on *P. finitimus* larvae and low on *A. andersoni*. This can be related to larval body size since *A. andersoni* have bigger larvae compared to *T. pyri* and *K. aberrans* (Croft *et al.*, 1999) and *P. finitimus* have the smallest ones (Duso C. personal observation).

In addition to prey consumption rate, the results suggest that predatory mites exhibit some preference in prey selection. Indeed, food preference is driven by its profitability in terms of energy content (Murdoch, 1969; van Baalen *et al.*, 2001) that can be estimated by the prey

conversion into egg biomass rate. *A. andersoni* females exhibited a higher performance on *T. pyri* than on the slightly smaller *K. aberrans*. This preference was underlined by the higher efficiency in prey conversion into egg biomass. Relationships between *A. andersoni* and *K. aberrans* do not suggest precise trends as reciprocal predation was less common than that exhibited on other heterospecifics and both species lived longer when fed with the other. The performance of *A. andersoni*, *K. aberrans* and *T. pyri* in terms of predation rate and fecundity proved to be better on *P. finitimus* larvae than on other prey. The low prey consumption and fecundity of the latter suggest that it is disadvantaged in interspecific predation. This is in agreement with the results obtained releasing *K. aberrans* in vineyards colonized by *P. finitimus*. However, *T. pyri* releases were not successful, suggesting that other factors could be involved in interspecific competition among these predatory mite species (Duso and Vettorazzo, 1999).

A comparison of the performance exhibited by predatory mites on a specific prey species offers another point of view of their potential impact in intraguild competition. *A. andersoni*, *K. aberrans* and *T. pyri* were more voracious than *P. finitimus*, *A. andersoni* consumed more *P. finitimus* larvae compared to *K. aberrans* and *T. pyri*, and *A. andersoni* preyed more on *T. pyri* larvae than *K. aberrans*. *Amblyseius andersoni* was the most aggressive among these species but its voracity did not imply higher fecundity rates; therefore this predator was the least efficient in converting prey food into egg biomass. Regarding the last two parameters *T. pyri* (and to a lesser extent *K. aberrans*) was superior to *A. andersoni*. Interactions between *A. andersoni* and *T. pyri* in field conditions did not follow precise trends. *Amblyseius andersoni* displaced *T. pyri* in some situations but the reverse was also observed (Croft, 1994, Lange and Trautman, 1994; Camporese and Duso, 1996). Cultivar features and in particular leaf morphology can mediate interactions between these two species: *T. pyri* was advantaged on cultivars with pubescent leaf undersurfaces whereas *A. andersoni* performed better on cultivars with glabrous or slightly hairy leaf undersurfaces (Duso and Vettorazzo, 1999).

Some interesting aspects emerged in the comparison between *K. aberrans* and *A. andersoni*: *K. aberrans* laid more eggs when *P. finitimus* was offered as prey and the fecundity of *A. andersoni* and *K. aberrans* did not differ on a diet based on *T. pyri* larvae. In both cases, prey conversion rate was higher for *K. aberrans* than for *A. andersoni*. This parameter could be considered as an indicator of the capacity for population persistence when prey is diminishing. *A. andersoni* is commonly observed in vineyards and orchards in Northern Italy but its population dynamics is subjected to fluctuations mainly due to adverse climate and lack of prey (e.g., Duso, 1989; Duso and Vettorazzo, 1999; Duso *et al.*, 2003). *K.*

*aberrans* populations show definite trends over the seasons (often related to pollen abundance) and are less subject to unpredictable fluctuations (Duso *et al.*, 1997, 2012). Problems encountered in managing *A. andersoni* populations suggested the release of *K. aberrans* in vineyards and apple orchards and this tactic proved to be an effective way to accelerate the establishment of this species and prevent spider mite outbreaks (Duso *et al.*, 2009; Duso *et al.*, 2012). In several cases competition between *K. aberrans* and *A. andersoni* has resulted in the dominance of the former species (Duso and Pasqualetto, 1993; Camporese and Duso, 1996; Duso *et al.*, 2009). Some factors affecting these results have been suggested but convincing evidence is lacking. Here we show that *K. aberrans* is advantaged over *A. andersoni* when interspecific predation involves larval stages. Additional studies also showed that *K. aberrans* is more efficient than *A. andersoni* in food conversion when predators are fed with the same spider mite prey (Lorenzon, 2012).

The theoretical framework of intraguild predation has been the subject of a consistent number of studies (Holt and Polis, 1997; Diehl and Feissel, 2000; Mylius *et al.*, 2001; Diehl, 2003; Tanabe and Namba, 2005). These studies indicate that coexistence is possible only if the intraguild prey is a more efficient at resource exploitation, but this state depends on the level of productivity (Polis and Holt 1992; Mylius *et al.*, 2001; Holt and Polis, 1997; Diehl and Feissel, 2000). Among the set of predatory mites considered here, the results suggest that *A. andersoni* should be considered as the intraguild predator. At the same time *A. andersoni* appeared to be the least efficient in food conversion, and conditions determining its dominance over other predatory mites' species occur only at high productivity level. This is consistent with other studies: at a high level of resource availability *A. andersoni* is able to displace other predatory mites' species while it can suffer from competition at low productivity level, tending to disperse from prey patches (Duso *et al.*, 2003; Walzer and Schausberger, 2011). Among the other species, *P. finitimus* appears to suffer from intraguild predation, and its efficiency in food conversion is not superior to that of *K. aberrans* and *T. pyri*. The differences in the profiles of *K. aberrans* and *T. pyri* are not clear. In previous investigations *T. pyri* was not clearly superior to *K. aberrans* in reciprocal predation (Schausberger, 1997, 1999a). Here we show that these two species have not marked differences in survival, fecundity and in food conversion efficiency when fed with reciprocal larvae. Some differences between *K. aberrans* and *T. pyri* emerged in juvenile development, since the latter species benefits more from predation upon heterospecifics than the former (Schausberger, 1999b). However, in field conditions, the interactions among *K. aberrans*, *T. pyri* and *P. finitimus* did not show precise trends while *K. aberrans* resulted often superior in

competition. A determinant role in the interactions among these predators is probably played by other factors such as indirect competition, environmental conditions and host-plant traits (e.g., Schausberger, 1998; 1997; Duso and Vettorazzo 1999; Lorenzon *et al.*, 2012).

The comparison between pollen and prey diets confirmed the positive effect of pollen on the fecundity of all four predatory mite species. Fecundity was higher on pollen than on predatory mite larvae but this is not surprising as some of them exhibited a higher fecundity on pollen than on tetranychid prey (e.g., Overmeer, 1985; Duso and Camporese, 1991; Schausberger, 1991, 1992; Engel and Ohnesorge, 1994; Kasap, 2005; Lorenzon *et al.*, 2012). This phenomenon has been reported for other predatory mites (e.g., McMurtry and Johnson, 1965; James, 1989; James and Whitney, 1993). In these studies an ad libitum supply of different food resources was offered. Thus it was expected that results would be affected by the quality of food sources. In the current work the number of prey consumed was largely lower than that of prey offered, suggesting that predatory mite response was also affected by food quality. Capacity for population increase on a defined food can contribute towards explaining why a predatory mite species becomes dominant in field conditions; in vineyards *A. andersoni* can be advantaged by mildew when coexisting with *K. aberrans* and *T. pyri* and laboratory studies showed that mildew is a more profitable food for *A. andersoni* than for the other two species (Duso *et al.*, 2003; Lorenzon *et al.*, 2012). Since the fecundity exhibited by the four predatory mites on pollen was similar we can suggest that this source of food does not greatly impact their interactions. In other papers pollen influenced interspecific interactions among predatory mites with outcomes depending on the initial size structure of competitors (Montserrat *et al.*, 2008). Further studies are needed to elucidate the role of pollen in mediating interspecific competition among generalist predatory mites.

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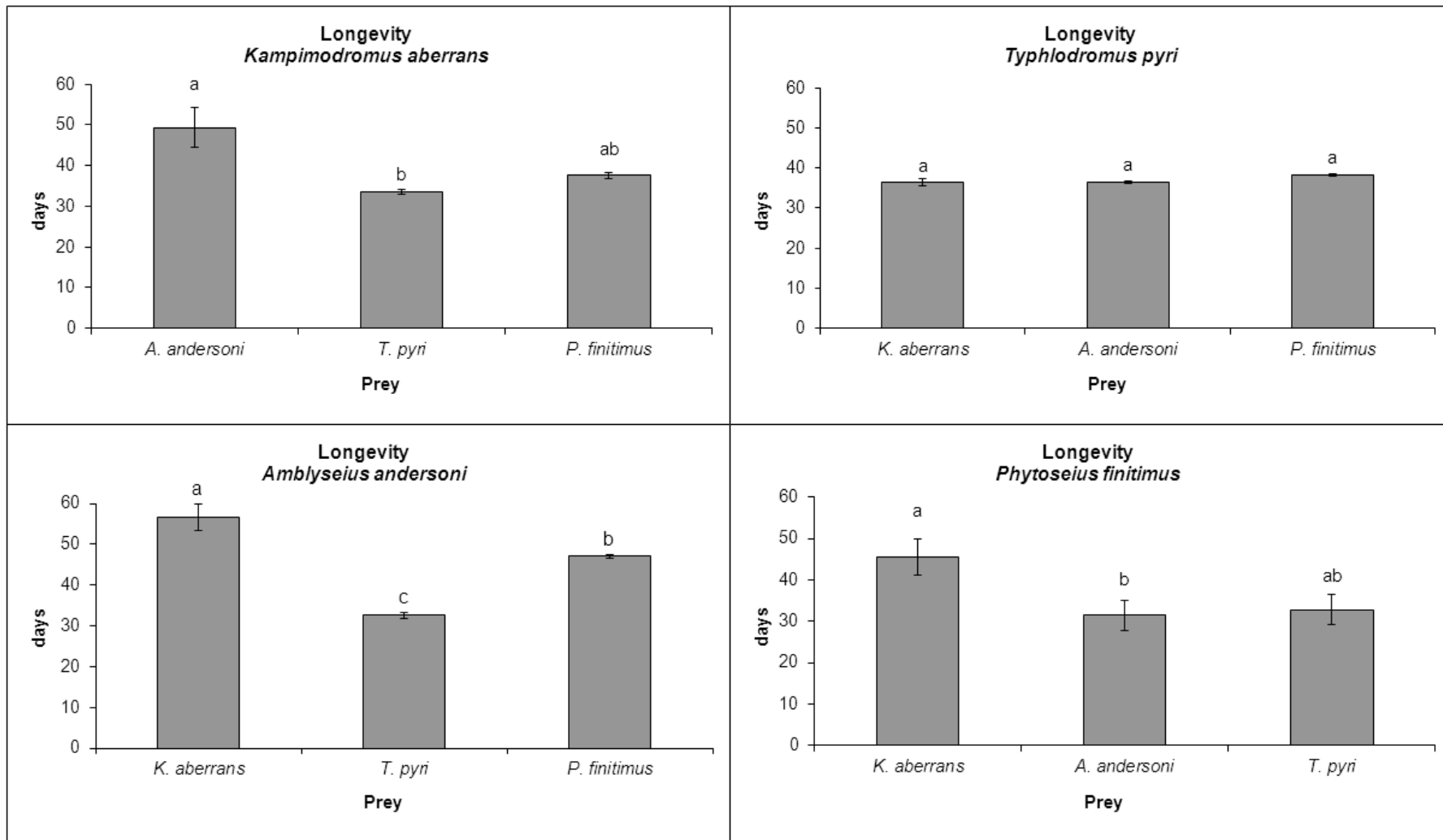


Figure 1 - Mean longevity ( $\pm$  SE) of adult females of *Kampimodromus aberrans*, *Amblyseius andersoni*, *Typhlodromus pyri* and *Phytoseius finitimus* when offered heterospecific larvae. Different letters indicate significant differences at Tukey-Kramer test ( $P = 0.05$ ).



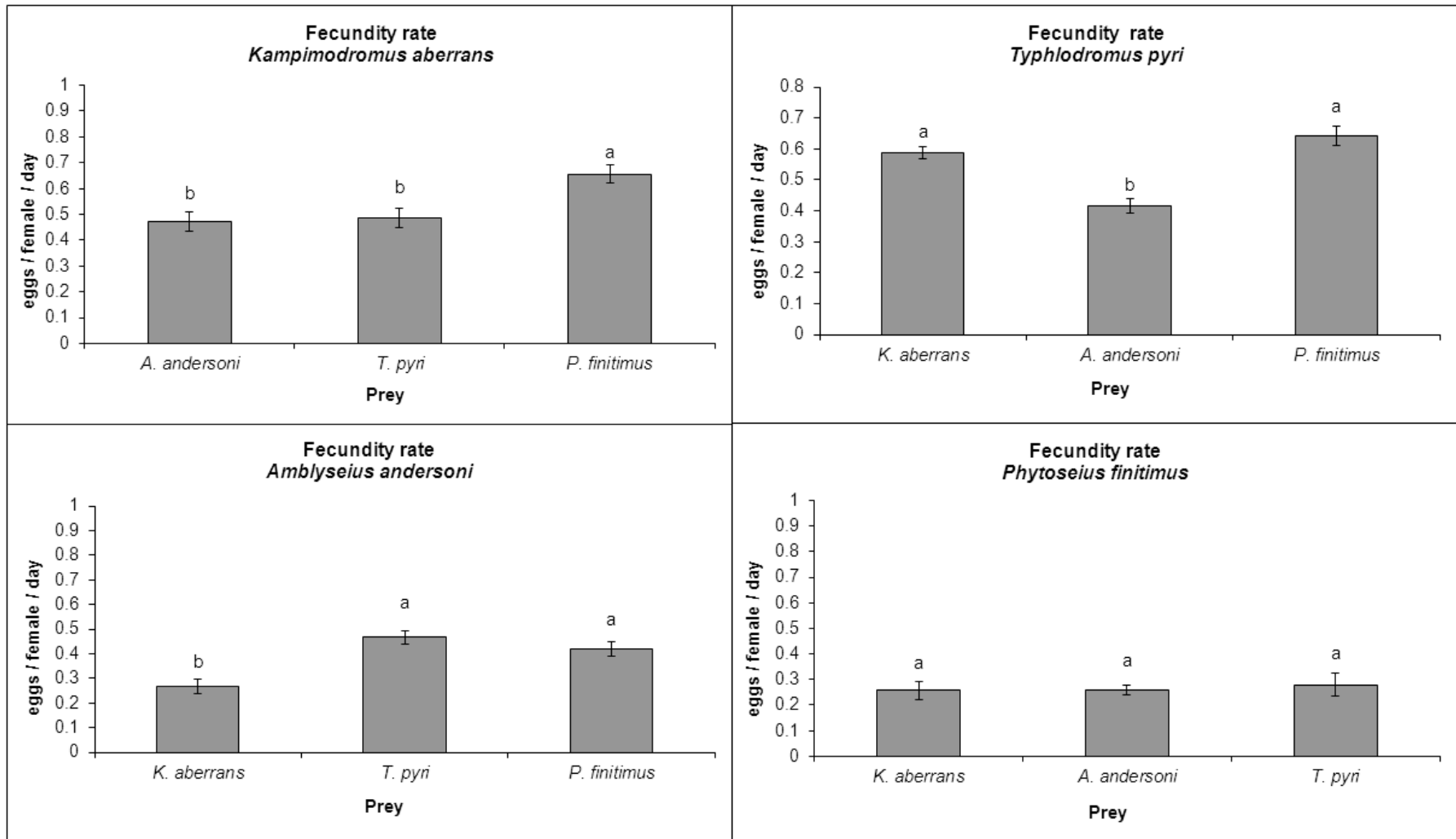


Figure 2 - Mean fecundity rate ( $\pm$  SE) of adult females of *Kampimodromus aberrans*, *Amblyseius andersoni*, *Typhlodromus pyri* and *Phytoseius finitimus* when offered heterospecific larvae. Different letters indicate significant differences at Tukey-Kramer test

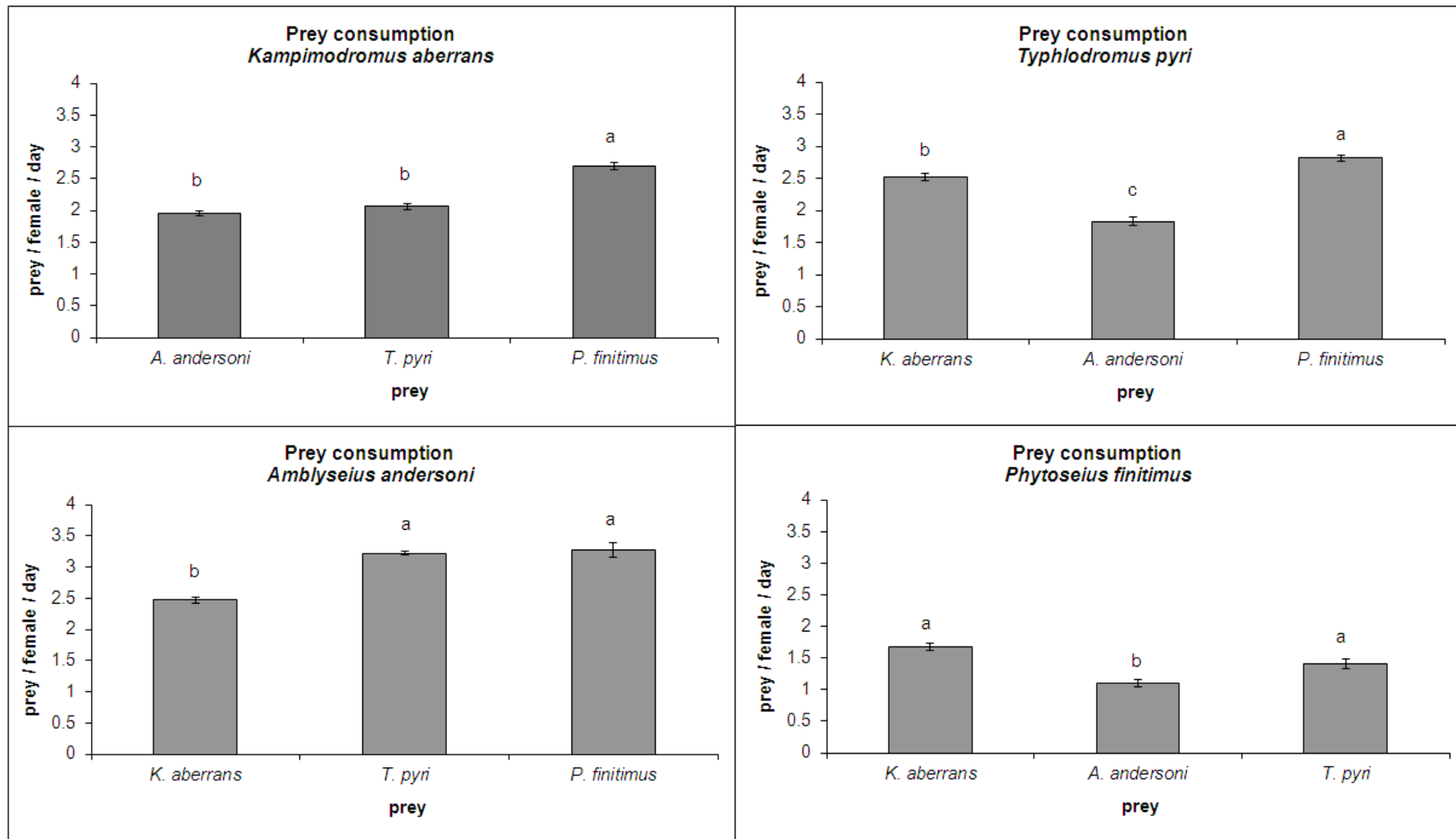


Figure 3 - Prey consumption rate ( $\pm$  SE) of adult females of *Kampimodromus aberrans*, *Amblyseius andersoni*, *Typhlodromus pyri* and *Phytoseius finitimus* when offered heterospecific larvae. Different letters indicate significant differences at Tukey-Kramer test (P = 0.05).

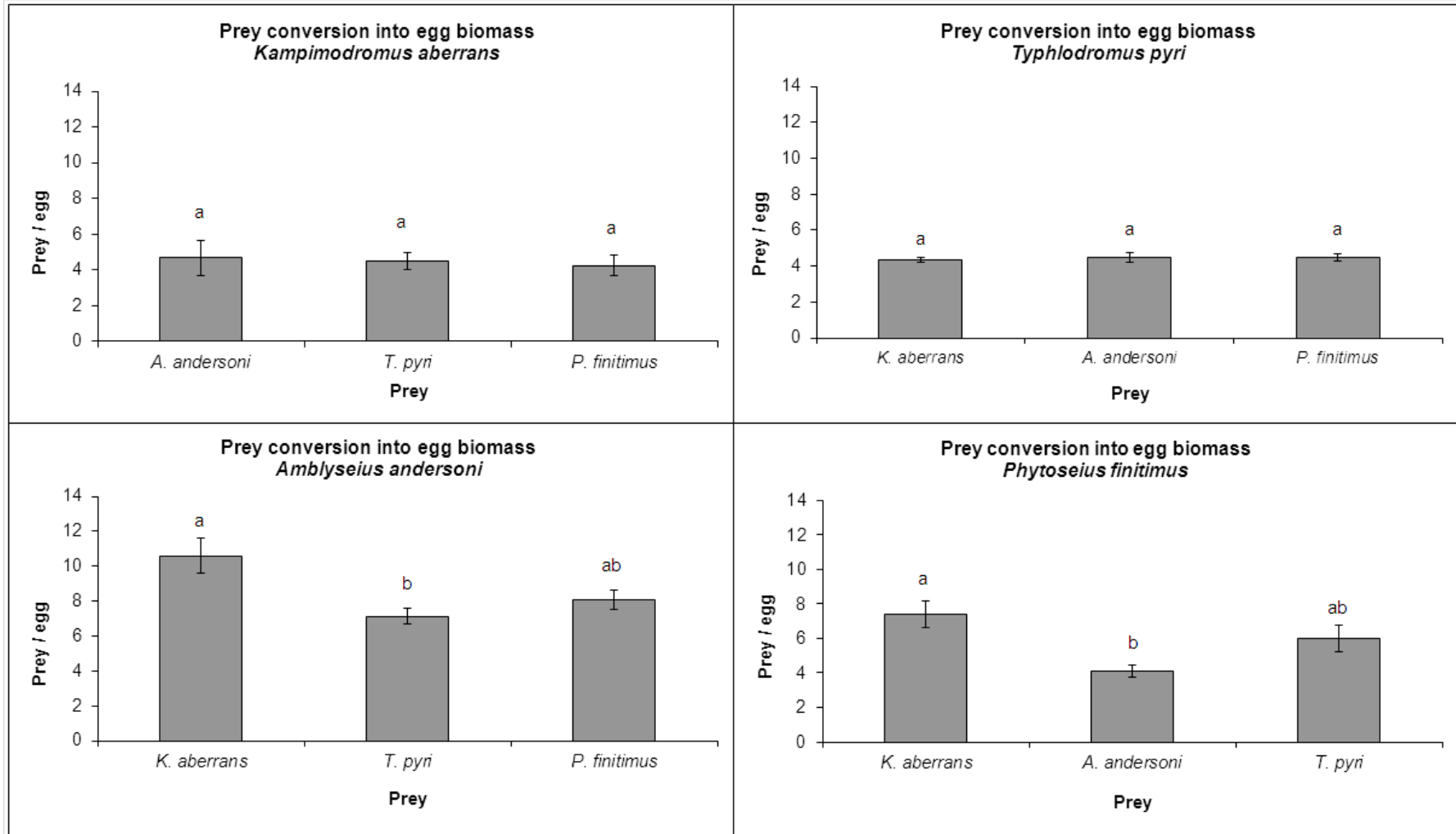


Figure 4 - Mean prey conversion rates ( $\pm$  SE) by *Kampimodromus aberrans*, *Amblyseius andersoni*, *Typhlodromus pyri* and *Phytoseius finitimus* when offered heterospecific larvae. Different letters indicate significant differences at Tukey-Kramer test ( $P = 0.05$ ).

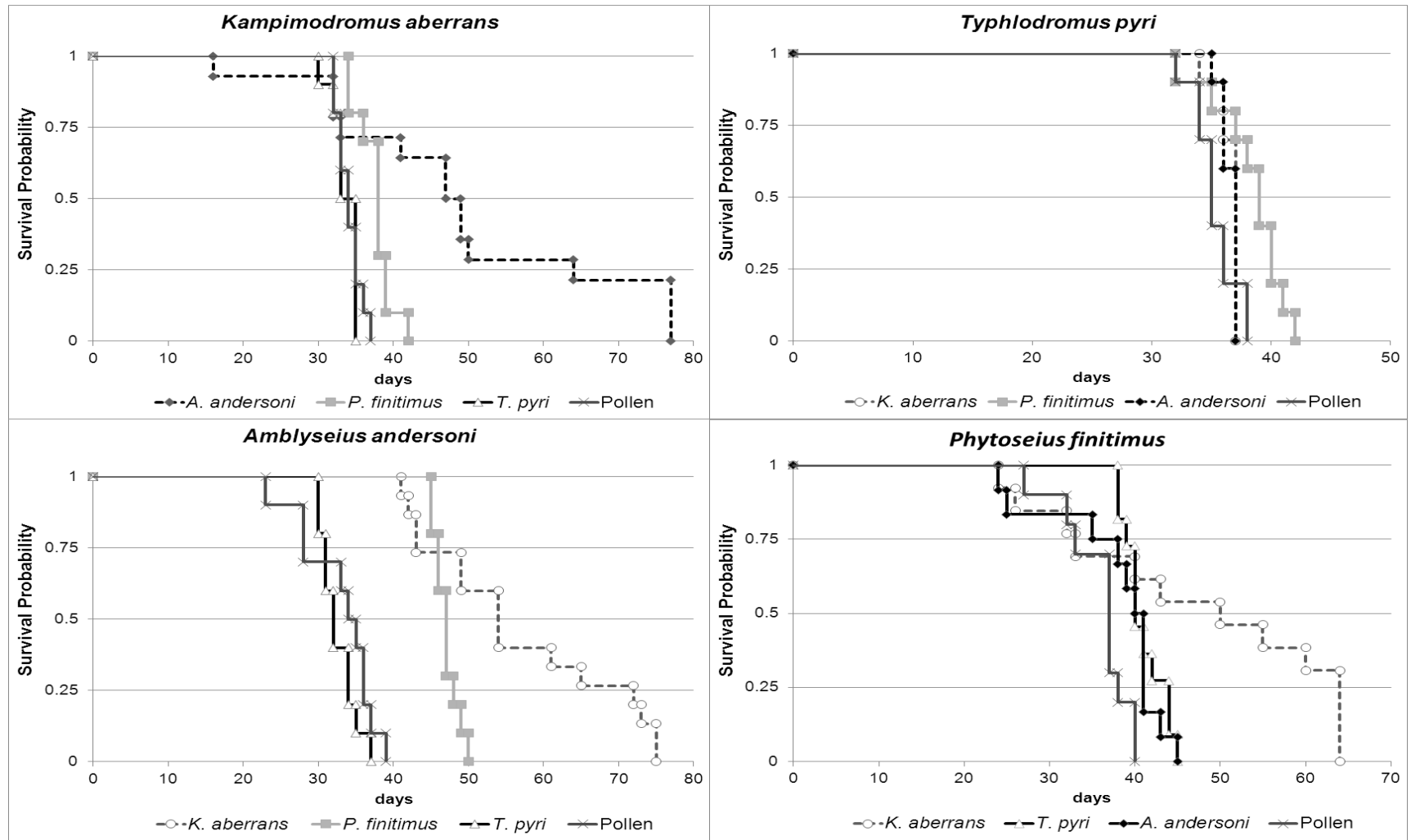


Figure 5 - Survival curves of *Kampimodromus aberrans*, *Typhlodromus pyri*, *Amblyseius andersoni* and *Phytoseius finitimus* females fed with different food sources.

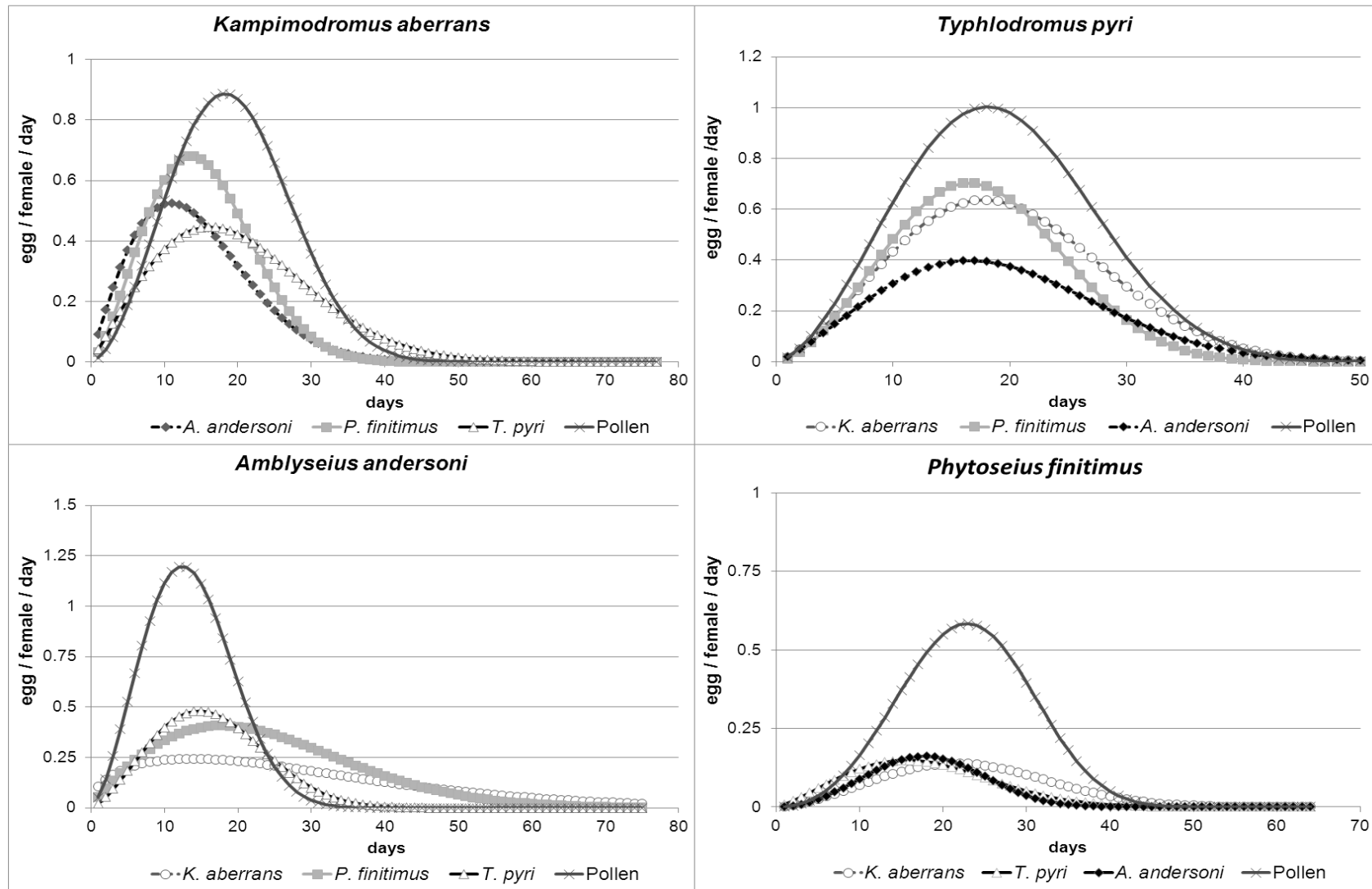


Figure 6 - Age-specific oviposition of *Kampimodromus aberrans*, *Typhlodromus pyri*, *Amblyseius andersoni* and *Phytoseius finitimus* females fed with different food sources estimated by Weibull density distribution model.

**Table 1** - Comparative longevity, fecundity, prey consumption and prey conversion rates ( $\pm$  SE) of predatory mite females fed with the same prey species. Different letters indicate significant differences at Tukey-Kramer test ( $P = 0.05$ ).

Predator	(No. Replicates)	Prey larvae	Longevity (Days)	Fecundity (eggs/female/day)	Prey consumption rate (prey/female/day)
<i>K. aberrans</i>	(15)	<i>A. andersoni</i>	49.36 $\pm$ 5.01 a	0.46 $\pm$ 0.04 a	1.95 $\pm$ 0.04 a
<i>T. pyri</i>	(10)	<i>A. andersoni</i>	36.50 $\pm$ 0.22 ab	0.42 $\pm$ 0.02 a	1.83 $\pm$ 0.07 a
<i>P. finitimus</i>	(15)	<i>A. andersoni</i>	31.40 $\pm$ 3.73 b	0.19 $\pm$ 0.03 b	1.10 $\pm$ 0.06 b
<i>K. aberrans</i>	(10)	<i>P. finitimus</i>	37.60 $\pm$ 0.76 b	0.66 $\pm$ 0.04 a	2.70 $\pm$ 0.05 b
<i>T. pyri</i>	(10)	<i>P. finitimus</i>	38.30 $\pm$ 0.94 b	0.64 $\pm$ 0.03 a	2.81 $\pm$ 0.05 b
<i>A. andersoni</i>	(10)	<i>P. finitimus</i>	47.00 $\pm$ 0.52 a	0.42 $\pm$ 0.03 b	3.27 $\pm$ 0.12 a
<i>T. pyri</i>	(10)	<i>K. aberrans</i>	36.40 $\pm$ 0.34 b	0.59 $\pm$ 0.02 a	2.51 $\pm$ 0.06 a
<i>A. andersoni</i>	(15)	<i>K. aberrans</i>	56.67 $\pm$ 3.26 a	0.27 $\pm$ 0.03 b	2.47 $\pm$ 0.04 a
<i>P. finitimus</i>	(15)	<i>K. aberrans</i>	45.50 $\pm$ 4.49 ab	0.26 $\pm$ 0.03 b	1.67 $\pm$ 0.06 b
<i>K. aberrans</i>	(10)	<i>T. pyri</i>	33.60 $\pm$ 0.54 a	0.49 $\pm$ 0.04 a	2.06 $\pm$ 0.04 b
<i>A. andersoni</i>	(10)	<i>T. pyri</i>	32.60 $\pm$ 0.73 a	0.47 $\pm$ 0.03 a	3.22 $\pm$ 0.03 a
<i>P. finitimus</i>	(15)	<i>T. pyri</i>	32.80 $\pm$ 3.70 a	0.27 $\pm$ 0.05 b	1.41 $\pm$ 0.08 c

**Table 2** - Survival of 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentiles with confidence limits (95 %) of predatory mites females fed with different food sources.

Predator	Food source	25th	CL(95%)		50th	CL(95%)		75th	CL(95%)	
		percentile	Upper	Lower	percentile	Upper	Lower	percentile	Upper	Lower
<i>K. aberrans</i>	Pollen	33	32	34	34	32	35	35	34	37
	<i>A. andersoni</i>	33	16	47	48	32	64	64	47	77
	<i>P. finitimus</i>	36	34	38	38	34	39	39	38	42
	<i>T. pyri</i>	33	30	35	34	30	35	35	33	35
<i>A. andersoni</i>	Pollen	28	23	35	34.5	23	36	36	34	39
	<i>K. aberrans</i>	43	41	54	54	43	65	72	54	75
	<i>P. finitimus</i>	46	45	47	47	45	48	48	47	50
	<i>T. pyri</i>	31	30	32	32	30	34	34	32	37
<i>T. pyri</i>	Pollen	34	32	35	35	34	36	36	35	38
	<i>K. aberrans</i>	36	34	37	37	36	37	37	-	-
	<i>P. finitimus</i>	37	32	39	39	37	40	40	39	42
	<i>A. andersoni</i>	36	35	37	37	36	37	37	-	-
<i>P. finitimus</i>	Pollen	33	27	37	37	27	38	38	37	40
	<i>K. aberrans</i>	33	24	50	50	32	64	64	43	64
	<i>T. pyri</i>	39	38	40	40	38	44	44	40	45
	<i>A. andersoni</i>	36.5	24	40	40.5	25	41	41	40	45

**Table 3** - Parameters and confidence intervals estimated by fitting the age-specific oviposition of *Kampimodromus aberrans*, *Typhlodromus pyri*, *Amblyseius andersoni* and *Phytoseius finitimus* females fed with different food sources to a Weibull density distribution model. All models fit the data ( $p < 0.01$ ). <sup>†</sup> Different letters indicate significant differences at Tukey – Kramer test ( $P = 0.05$ ). \* Pseudo- $R^2$  values were calculated according to Schabenberger (1998).

	<sup>a</sup> Eggs <sub>mx</sub> (Eggs)	A	CI (95%)		B	CI (95%)		<sup>b</sup> Pseudo - R <sup>2</sup>	
			Lower	Upper		Lower	Upper		
<i>Kampimodromus aberrans</i>									
Pollen	18.1 ± 0.546 a	0.00027	0.00001	0.00052	2.672	2.366	2.979	0.25	
<i>A. andersoni</i>	10 ± 1.153 b	0.00479	0.0002	0.00939	1.928	1.585	2.271	0.79	
<i>P. finitimus</i>	12.1 ± 0.766 b	0.00117	0.00004	0.00231	2.372	2.037	2.707	0.42	
<i>T. pyri</i>	11.6 ± 0.832 b	0.00174	-0.00047	0.00395	2.038	1.624	2.453	0.53	
<i>Amblyseius andersoni</i>									
Pollen	18.2 ± 0.904 a	0.00113	0.00037	0.00189	2.489	2.245	2.732	0.41	
<i>K. aberrans</i>	10.4 ± 0.965 bc	0.00707	-0.00065	0.0148	1.437	1.107	1.767	0.87	
<i>P. finitimus</i>	13.3 ± 0.651 b	0.00213	-0.00032	0.00459	1.87	1.513	2.228	0.61	
<i>T. pyri</i>	9.2 ± 0.489 c	0.00097	-0.00018	0.00211	2.375	1.975	2.774	0.54	
<i>Typhlodromus pyri</i>									
Pollen	20.8 ± 0.8 a	0.00032	0.00006	0.00058	2.616	2.355	2.876	0.29	
<i>K. aberrans</i>	14.2 ± 0.611 b	0.00052	-0.00005	0.00109	2.442	2.088	2.796	0.33	
<i>P. finitimus</i>	13 ± 0.557 b	0.00035	-0.00001	0.00071	2.672	2.328	3.016	0.31	
<i>A. andersoni</i>	9.1 ± 0.604 c	0.00097	-0.00023	0.00217	2.266	1.865	2.667	0.61	
<i>Phytoseius finitimus</i>									
Pollen	11.6 ± 0.945 a	0.00002	-0.00002	0.00006	3.313	2.783	3.844	0.42	
<i>K. aberrans</i>	3.5 ± 0.344 b	0.00022	-0.00041	0.00085	2.581	1.715	3.448	0.9	
<i>T. pyri</i>	3.2 ± 0.416 b	0.00112	-0.00159	0.00384	2.261	1.459	3.064	0.89	
<i>A. andersoni</i>	2.8 ± 0.489 b	0.00012	-0.00019	0.00043	2.991	2.159	3.823	0.42	



## Chapter IV

### **The impact of insecticides applied in apple orchards on the predatory mite *Kampimodromus aberrans* (Acari: Phytoseiidae)**

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In this work, I contributed to laboratory and field experiments 2010

## **Abstract**

*Kampimodromus aberrans* is an effective predatory mite in fruit orchards. The side-effects of insecticides on this species have been little studied. Field and laboratory experiments were conducted to evaluate the effects of insecticides on *K. aberrans*. Field experiments showed the detrimental effects of etofenprox, tau-fluvalinate and spinosad on predatory mites. Spider mite populations reached higher densities on plots treated with etofenprox and tau-fluvalinate than in the other treatments. Single or multiple applications of neonicotinoids caused no detrimental effects on predatory mites. In the laboratory, spinosad and tau-fluvalinate caused 100% mortality. Etofenprox caused a significant mortality and reduced fecundity. The remaining pesticides did not affect female survival except for imidacloprid. Thiamethoxam, clothianidin, thiacloprid, chlorpyrifos, lufenuron and methoxyfenozide were associated with a significant reduction in fecundity. No effect on fecundity was found for indoxacarb or acetamiprid. Escape rate resulted as relatively high for etofenprox and spinosad, and to a lesser extent thiacloprid. The use of etofenprox, tau-fluvalinate and spinosad resulted as detrimental for *K. aberrans* and the first two pesticides induced spider mite population increases. The remaining pesticides caused no negative effects on predatory mites. Some of them (reduced fecundity and repellence) should be considered in IPM tactics.

## **Introduction**

Mating disruption is widely used to control the codling moth *Cydia pomonella* L. in several European fruit growing areas but insecticides are still needed to control a number of apple pests (e.g., aphids, leafminers and scales). Mating disruption is not applicable in a number of situations where insecticides remain the most frequent control measure against *C. pomonella*. A high number of fungicide applications are required to control apple scab, powdery mildew and other diseases in apple orchards. Multiple pesticide treatments may therefore be applied on these crops despite efforts to reduce them following Integrated Pest Management strategies. Knowledge of pesticide side-effects on beneficials is essential to preserve their populations in a defined crop system and reduce risks from pest outbreaks. Phytophagous mites (e.g., Tetranychoidae and Eriophyoidea) are very sensitive to pesticide use and can build up large populations when natural enemy's populations are destroyed by non-selective chemicals. Predatory mites belonging to the Phytoseiidae family are key-biocontrol agents of phytophagous mites in orchards. Some predatory mite species have been considered as representative non-target organisms in perennial crops and a number of testing programmes have been undertaken to evaluate the effects of several pesticides on them in laboratory, semi-

field and field conditions (e.g., Sterk *et al.*, 1999; Bostanian *et al.*, 2009). Pest control strategies and mite fauna differ from one geographic region to another, for instance in southern and central European orchards or vineyards. Studies on the compatibility of pesticides with conservation biological control tactics based on predatory mites should be developed at a regional level considering potentially involved factors (Bostanian *et al.*, 2010; Lefebvre *et al.*, 2011, 2012). Predatory mites can be released artificially in orchards using inexpensive techniques to accelerate biological control processes and the release of pesticide resistant predatory mites has attracted a number of researchers who have obtained promising results (e.g., Baillod and Guignard, 1984; Prokopy and Christie, 1992; Solomon *et al.*, 1993; Blommers, 1994; Lester *et al.*, 2000; Marshall *et al.*, 2001; Jung *et al.*, 2004; Duso *et al.*, 2009). Among predatory mites occurring in Europe *Kampimodromus aberrans* (Oudemans) proved to be effective in controlling phytophagous mite populations in vineyards (Ivancich Gambaro, 1973; Duso, 1989; Girolami *et al.*, 1992; Kreiter *et al.*, 2000; Tixier *et al.*, 1998; Duso *et al.*, 2012). However, its potential proved to be limited for a long time because of its susceptibility to several pesticides, mainly Ethylene-bis-dithiocarbamate (EBDC) fungicides and broad-spectrum insecticides such as organophosphates (OPs) (Ivancich Gambaro, 1973; Girolami, 1987; Pozzebon *et al.*, 2002). *Kampimodromus aberrans* has also been detected in other agricultural systems, mainly hazelnuts and apples (e.g., Garcia-Mari *et al.*, 1989; Fischer-Colbrie and El Borolossy, 1990; Espinha *et al.*, 1995; Nicotina and Cioffi, 1998; Tsolakis *et al.*, 2000; Kasap, 2005). In most of these studies *K. aberrans* was detected in untreated orchards or with a low pesticide pressure. While commercial hazelnuts are not subjected to many pesticide treatments, the number of insecticide applications per year in conventional apple orchards can be significant (e.g., 6-10 per year in Northern Italy if mating disruption is not adopted, see Angeli *et al.*, 2007). Strains resistant to conventional pesticides (OPs, carbamates, pyrethroids) in European fruit orchards have been reported in two predatory mite species, i.e. *Typhlodromus pyri* Scheuten and *Amblyseius andersoni* (Chant) since the 1970s and this can explain their dominance over heterospecifics (e.g., Ivancich Gambaro, 1975; Van de Baan *et al.*, 1985; Anber and Overmeer, 1988; Duso, 1992; Duso *et al.*, 1992; Blommers, 1994; Baillod and Guignard, 1984; Fitzgerald and Solomon, 2000, 2002). Strains of *K. aberrans* apparently resistant to OPs and EBDC fungicides have been reported to occur in some vineyards located in northern Italy (Corino *et al.*, 1986; Vettorello and Girolami, 1992; Posenato, 1994). Resistance to EBDCs was then demonstrated definitively in France (Auger *et al.*, 2004) and to OPs in Italy (Tirello *et al.*, 2012). Climate change in the last decades resulted in unusually high temperatures in Italian subalpine areas

(e.g. Trentino) and these conditions seemed to affect both *A. andersoni* and *T. pyri* persistence and performance (Angeli, pers. comm.). *K. aberrans* proved to persist in conditions of prey scarcity and high temperatures and to compete with these predatory mite species (Duso and Vettorazzo, 1999). Therefore *K. aberrans* was selected for experimental releases in Trentino apple orchards. A strain originating from the neighbouring Veneto region and apparently resistant to various pesticides (Posenato, 1994) was introduced in a number of commercial apple orchards located in the Trentino region in the late 1990s. Releases were successful and *K. aberrans* displaced native predatory mites (including *T. pyri* and *A. andersoni*) despite the application of various insecticides and fungicides (Duso *et al.*, 2009). In this and other areas broad-spectrum insecticides have recently been replaced by insecticides with a lower risk for human health, e.g. some insect growth regulators (IGRs) and neonicotinoids. However, the impact of these products on predatory mites is not well investigated (e.g., see Bostanian *et al.*, 2009, 2010 for North-American species). Field experiments were conducted to evaluate the effects of insecticides frequently applied in European apple orchards on this *K. aberrans* strain. At the same time laboratory studies aimed at assessing lethal and sub-lethal effects of these pesticides were conducted to provide information useful for improving Integrated Pest Management (IPM) strategies.

## **Materials and Methods**

### **Field studies**

The effects of a number of insecticides on *K. aberrans* populations were evaluated in an apple orchard located at the experimental station of E. Mach Foundation (FEM, S. Michele all'Adige, Trento, Italy) in the 2009 and 2010 growing seasons. The orchard surface area was approximately 5000 m<sup>2</sup> and contained 12 years old Golden Delicious plants. Seven insecticides commonly applied in apple orchards in Europe and elsewhere were considered: chlorpyrifos, thiacloprid, acetamiprid, lufenuron, indoxacarb, methoxyfenozide and etofenprox.

Insecticides were applied according to codling moth control timing. In 2009, they were applied on 18 June and 15 July; acetamiprid was used on the first date only. In 2010, pesticide applications were on 25 May and 25 June. A randomized block design was adopted with four replicates per treatment. Sampling was conducted before and approximately every 10 days after insecticide applications (until 12 August 2009, and 21 July 2010). A total of 60 leaves per treatment (15 leaves per replicate) were removed and transferred to the laboratory where

predatory and phytophagous mites, eventually present, were counted under a dissecting microscope. The phytoseiids were mounted on slides, in Hoyer's medium, and identified under a phase contrast microscope. Data were analyzed with a Restricted Maximum Likelihood (REML) repeated measures model with the SAS MIXED procedure (SAS Institute Inc., 1999). Mite densities were considered as response variables with repeated measures made at different times, i.e. sampling dates. Using an F test ( $\alpha = 0.05$ ) we evaluated the effect of insecticide application, time and their interaction. Contrasts ( $\alpha = 0.05$ ) were designed for pairwise comparison between treatments before and after insecticide applications. Degrees of freedom were estimated using the Kenward-Roger method (Littell *et al.*, 1996). According to Akaike's Information Criterion, first-order autoregressive was chosen as best fitting covariance structure for correlating different sampling dates (Littell *et al.*, 1996). Data were checked for analysis assumptions and square-root transformation was applied.

Two additional experiments were performed in 2011 in other apple orchards located in Trentino. In the first experiment, the effects of clothianidin, thiacloprid, imidacloprid, tau-fluvalinate, thiamethoxam and acetamiprid were compared on *K. aberrans* populations. An untreated control was included for a comparison. The number of insecticide applications (1, 2 or 3) was considered as nested effect within each insecticide treatment. The overall experimental design resulted in 20 treatments. Insecticides were applied on 23 May, 22 June, and 21 July. A completely randomized block design with three replicates per treatment was adopted. In the second experiment, spinosad and etofenprox were compared with an untreated control to assess their impact on *K. aberrans* populations. Insecticides were applied on 8 June. A completely randomized block design with four replicates per treatment was used. Leaves and mites were processed as in the previous experiment. Data of 2011 experiments were analyzed with a Restricted Maximum Likelihood (REML) repeated measures model with treatments; times and their interactions were considered as sources of variation and F tests were used to evaluate their effects ( $\alpha = 0.05$ ). Degrees of freedom were estimated using the Kenward-Roger method (Littell *et al.*, 1996). Mite densities were considered as response variables with repeated measurements at different times, i.e. sampling dates. Data were checked for normality assumption and thus the number of phytoseiids per leaf was  $\log(x+1)$  transformed prior to the analyses. The SLICE option of the LSMEANS statement was used to test treatment effect variation during observation periods (SAS Institute Inc., 1999). For the first experiment of 2011 differences among treatments were evaluated with a t-test with Bonferroni adjustment ( $\alpha = 0.05$ ) to least square means, while for the second experiment pairwise comparison between treatments in the period after treatment were performed using

contrasts ( $\alpha = 0.05$ ).

## Laboratory studies

Pesticides applied in field trials were tested at the same concentrations in the laboratory. Apple leaves were treated with pesticides and then young *K. aberrans* females were transferred onto the leaves to expose them to fresh pesticide residues. The detailed experimental procedure is reported in Tirello *et al.*, (submitted). Fresh pollen was provided every two days as food for predatory mites. The experimental units were kept in a climatic chamber at  $25 \pm 2$  °C,  $70 \pm 10\%$  relative humidity and 16L: 8D photoperiod. Effect of pesticides on female mortality was evaluated after 72 h. Surviving females were observed daily for additional four days to assess effects on fecundity. Eggs' hatching was also monitored until 100 % hatching rate was reached in the control. Escaped or drowned females were removed from the initial number. In total we assessed 45-50 females per pesticide.

We performed one-way ANOVA with F test ( $P = 0.05$ ) to evaluate the effect of pesticides on mite survival, fecundity, escape rate and egg hatching using GLM procedure of SAS<sup>40</sup>. Treatments were compared using Tukey–Kramer test ( $P = 0.05$ ). In order to meet the ANOVA assumptions, data on survival were arcsin-transformed while square-root transformation was applied to data on fecundity. The Blümel and Hausdorf<sup>43</sup> formula was used for fecundity calculation. The overall toxicity of each pesticide was expressed as:

$$E = 100\% - (100\% - M) \cdot R$$

Where E is the coefficient of toxicity; M is the corrected mortality according to Abbott (1925); R is the ratio between the average number of hatched eggs produced by treated females and the average number of hatched eggs produced by females in the control group.

## Results

### Field studies

In 2009, the first insecticide application affected *K. aberrans* populations ( $F_{7, 50.1} = 6.94$ ;  $P < 0.001$ ). No differences were observed among treatments prior to insecticide applications ( $F_{7, 140} = 1.85$ ;  $P = 0.081$ ). Later, predatory mite numbers were significantly reduced by etofenprox compared to other treatments ( $P < 0.01$  in all comparisons; Figure 1). There were no differences among treatments before the second insecticide application ( $F_{6, 99.8} = 1.74$ ;  $P = 0.119$ ) but they emerged on the subsequent sampling dates ( $F_{6, 37.3} = 3.07$ ;  $P = 0.015$ ; Figure. 1). Lower *K. aberrans* numbers were recorded in etofenprox treated plots compared to the control ( $F_{1, 40.8} = 5.22$ ;  $P = 0.027$ ), chlorpyrifos ( $F_{1, 40.8} = 4.27$ ;  $P = 0.045$ ) and indoxacarb ( $F$

$F_{1, 40.8} = 10.17$ ;  $P = 0.002$ ). Higher predatory mite densities were observed in indoxacarb compared to methoxyfenozide plots ( $F_{1, 40.8} = 6.28$ ;  $P = 0.016$ ). No differences were observed among etofenprox, lufenuron, thiacloprid and methoxyfenozide ( $P > 0.05$  in all comparisons). Among phytophagous mites, the presence of *Panonychus ulmi* (Koch) was detected. *Panonychus ulmi* populations reached low levels with no effect of insecticides ( $F_{7, 56.3} = 0.46$ ;  $P = 0.862$ ;  $F_{7, 46.2} = 0.97$ ;  $P = 0.471$ ; after the first and second application respectively Figure 1).

In 2010, *K. aberrans* populations increased from May to June when relatively high densities were reached. The first insecticide application exerted significant effects on *K. aberrans* populations ( $F_{7, 21.7} = 21.96$ ;  $P < 0.001$ ). Predatory mite numbers dramatically declined after the use of etofenprox ( $P < 0.001$  in all comparisons; Figure 2). There were no significant effects when the remaining insecticides were applied ( $P > 0.15$  in all comparisons). A significant effect of insecticide application was observed on *P. ulmi* ( $F_{1, 38.7} = 2.93$ ;  $P = 0.015$ ), which reached higher densities on etofenprox treated plants ( $P < 0.01$  in all comparisons; Figure 2). No effect of the other insecticides was observed ( $P > 0.4$  in all comparisons). An additional insecticide treatment was applied in late June. Considering the overall season, the effect of the two insecticide treatments on *K. aberrans* populations was significant ( $F_{7, 50} = 51.94$ ;  $P < 0.001$ ), and etofenprox was confirmed to be the most detrimental ( $P < 0.001$  in all comparisons). No differences emerged among other insecticides ( $P > 0.10$  in all comparisons). At the same time *P. ulmi* densities were significantly higher in etofenprox plots than in other treatments ( $F_{7, 62.5} = 18.80$ ;  $P < 0.001$ ;  $P < 0.0001$  in all comparisons). No differences were found among the others treatments ( $P > 0.4$  in all comparisons).

In the first experiment of 2011, a significant variation in *K. aberrans* numbers was found among treatments ( $F_{18, 321} = 42.34$ ;  $P < 0.0001$ ) and over time ( $F_{9, 321} = 36.03$ ;  $P < 0.0001$ ). There was also a significant interaction between treatments and time ( $F_{162, 286} = 2.10$ ;  $P < 0.0001$ ). No differences among treatments were found prior to the first insecticide application ( $F_{18, 373} = 1.13$ ;  $P = 0.324$ ) while significant differences emerged later (Table 2; Figure 3). In particular, insecticide applications determined low numbers of predatory mites compared to the control, independently of application frequency (Table 2; Figure 3). Among insecticides, tau-fluvalinate was the most detrimental to predatory mites (Table 2; Figure 3). *Panonychus ulmi* was present and its population level differed among treatments ( $F_{18, 111} = 6.00$ ;  $P < 0.0001$ ) and sampling times ( $F_{9, 331} = 21.89$ ;  $P < 0.0001$ ), and a significant interaction “treatment\*time” was found ( $F_{162, 292} = 3.63$ ;  $P < 0.0001$ ). There was no effect of treatments

before the first insecticide application ( $F_{18, 340} = 0.05$ ;  $P = 1$ ) but later higher spider mite densities were associated with applications of tau-fluvalinate (Table 3).

In the second experiment of 2011, insecticide application was associated with a decrease in predatory mite numbers ( $F_{2, 11.1} = 29.82$ ;  $P < 0.0001$ ; Figure 4). *Kampimodromus aberrans* density changed over time ( $F_{4, 33.5} = 26.67$ ;  $P < 0.0001$ ) and a significant interaction ‘treatment\*time’ was found ( $F_{8, 33.8} = 3.94$ ;  $P = 0.002$ ). There were no differences among treatments prior to insecticide application ( $F_{2, 33.5} = 0.21$ ;  $P = 0.81$ ). After insecticide treatment, higher levels of predatory mites were found in the control compared to spinosad ( $F_{1, 12.6} = 57.18$ ;  $P < 0.0001$ , Figure. 4) and etofenprox ( $F_{1, 12.6} = 55.73$ ;  $P < 0.0001$ , Figure 4). No differences were found between spinosad and etofenprox ( $F_{1, 12.6} = 0.01$ ;  $P = 0.925$ , Figure. 4). Low *P. ulmi* densities were detected without differences among treatments ( $F_{2, 9.31} = 0.97$ ;  $P = 0.413$ ; Figure. 4).

### Laboratory studies

Pesticides affected *K. aberrans* survival ( $F_{11, 503} = 133.80$ ;  $P < 0.0001$ ; Figure 5) and fecundity ( $F_{9, 258} = 21.76$ ;  $P < 0.0001$ ; Figure 5). Spinosad and tau-fluvalinate caused 100% mortality. The two formulations of etofenprox caused 90.7% and 58.2% mortality respectively and survived females manifested lower fecundity (Table 2). The remaining pesticides did not affect female survival except for imidacloprid, where a survival of 81.7% was observed along with a low level of fecundity (Figure. 5, Table 2). Thiamethoxam, clothianidin, thiacloprid, chlorpyrifos, lufenuron and methoxyfenozide were associated with a significant reduction in fecundity (Figure 5, Table 2). No effect on fecundity was found for indoxacarb and acetamiprid (Figure 5).

Escape rate was also affected by pesticides ( $F_{11, 519} = 5.29$ ;  $P < 0.0001$ ), resulting as relatively high for the two formulations of etofenprox, spinosad, and to a lesser extent thiacloprid. Escape rates of other pesticides were not significantly different compared to the control (Figure5).



## Discussion

In field trials etofenprox and tau-fluvalinate applications were associated with significant effects on *K. aberrans* densities. Data on etofenprox confirm previous observations conducted on *K. aberrans* in vineyards (Girolami *et al.*, 2001; Tosi *et al.*, 2006). The detrimental effects of tau-fluvalinate on predatory mites also confirm the results obtained in other investigations (Petitt and Karan, 1991; Bellows *et al.*, 1992; Grout *et al.*, 1997; Amin *et al.*, 2009). Our laboratory study shows that these effects involve essentially the survival of predatory mites. The effects of etofenprox on females exposed to fresh pesticide residues were comparable with those reported for *Neoseiulus longispinosus* (Evans) (Kongchuensin and Takafuji, 2006). In the latter study the detrimental effects of etofenprox declined when the predatory mite was exposed to aged residues. We also showed a repellent activity of etofenprox on *K. aberrans* in the laboratory. Lethal and sub-lethal effects of tau-fluvalinate and etofenprox on *K. aberrans* females were associated with spider mite increases in the experimental plots. It should be stressed that pyrethroid residues also induce sub-lethal effects on spider mites, e.g. increasing locomotory activity or escape (Bowie *et al.*, 2001; Holland and Chapman, 1994). Field concentrations of pyrethroids can disrupt predator-prey dynamics in apple orchards (Bostanian *et al.*, 1985; Bowie *et al.*, 2001) and this was observed in our trials. The residual toxicity of pyrethroids on predatory mites can differ among active ingredients, that of etofenprox being lower than that of cypermethrin (Kongchuensin and Takafuji, 2006). These differences may have practical implications in field conditions.

Spinosad exerted dramatic effects on *K. aberrans* populations and this was likely due to its impact on predatory mite survival. A significant escape rate was also observed with spinosad suggesting its repellence to predatory mites exposed to foliar residues. Investigations on the effects of spinosad on different predatory mite species gave contrasting results (e.g. Williams *et al.*, 2003; Kim *et al.*, 2005; Van Driesche *et al.*, 2006; Villanueva and Walgenbach, 2005; Yoo and Kim, 2000; Ahn *et al.*, 2004). However, studies conducted in conditions similar to those in the present study showed significant effects of spinosad on predatory mites. An experimental work conducted in a vineyard showed 54-60% reduction in *K. aberrans* population size 10 and 20 days after spinosad treatment, respectively (Tosi *et al.*, 2006). In another field trial *T. pyri* population density was reduced by 43% by one spinosad application (Miles and Dutton, 2003). Our laboratory results highlighted the toxic effects of spinosad in the laboratory at 14.4 g a.i. hl<sup>-1</sup>. The response to spinosad by other predatory mites (e.g. *Phytoseiulus persimilis* Athias-Henriot) in the laboratory appeared to be less dramatic than that seen for *K. aberrans* (Williams *et*

*al.*, 2003). Duso *et al.*, (2008) tested spinosad at 36 g a.i. hl<sup>-1</sup> and found 47% mortality of *P. persimilis* females along with a 57% reduction in oviposition. The possibility of combining *P. persimilis* releases and spinosad applications has been explored with some positive results (e.g. Ahn *et al.*, 2004; Holt *et al.*, 2006; Miles and Dutton, 2003). This is probably due to the relatively low persistence of spinosad. Direct exposure to spinosad resulted in >90% mortality in various predatory mites but this effect was dramatically reduced when aged residues were considered (Miles and Dutton, 2003; Kongchuensin and Takafuji, 2006; Bernard *et al.*, 2010; Rahman *et al.*, 2011). The compatibility of spinosad with predatory mite releases depends on the time after pesticide application. This is more important for protected crops than for perennial ecosystems where conservation biological control is crucial and the use of unselective pesticides can cause serious problems with spider mites for a long time. Field data reported in this work show a significant impact of a single application of spinosad on *K. aberrans* populations. This could have much more importance when multiple applications of spinosad are planned, such as on organic farms where pesticide use is restricted to few compounds. Repeated applications of spinosad on organic apple orchards were associated with failures of *K. aberrans* releases (S. Ahmad, unpub. data) and semi-field experiments conducted on the same predatory mite confirmed this detrimental effect (Pozzebon *et al.*, unpub. data).

In the present study, neonicotinoids exhibited some effects on *K. aberrans* in field conditions but the response of the predatory mites differed among trials. There were no effects on spider mite outbreaks after single or multiple applications of neonicotinoids in contrast with previous findings in North America (Beers *et al.*, 2005). Our laboratory studies showed a low effect of neonicotinoids on *K. aberrans* survival confirming the results of experiments conducted on other predatory mites (Mizell and Sconyer, 1992; James, 1997; James and Vogele, 2001; Poletti *et al.*, 2007; Lefebvre *et al.*, 2011). In other studies imidacloprid (0.13 g a.i. l<sup>-1</sup>) was more toxic to *G. occidentalis* and *N. fallacis* than to *A. andersoni* when predatory mites were exposed directly to the pesticide (James, 2003). Lower rates were still highly toxic to *G. occidentalis* and *N. fallacis*. Imidacloprid affected the latter species even through systemic and residual routes of exposure. Bostanian *et al.*, (2009) reported a high toxicity of imidacloprid and acetamiprid to *G. occidentalis* adult females whereas thiamethoxam and thiacloprid showed slight or negligible effects. In the current study neonicotinoids significantly affected *K. aberrans* fecundity. A similar effect was reported for *N. californicus* exposed to imidacloprid (Castagnoli *et al.*, 2005), *N. fallacis* exposed to thiamethoxam (Villanueva and Walgenbach, 2005), and *G. occidentalis*

exposed to imidacloprid, acetamiprid and to a lesser extent thiamethoxam (Bostanian *et al.*, 2009). Neonicotinoids (i.e. imidacloprid, thiamethoxam, acetamiprid, and thiacloprid) have been reported to cause repellence to predatory mites (Bostanian *et al.*, 2009) and some of them (i.e. acetamiprid, imidacloprid and thiamethoxam) alter the functional response of *N. californicus* and *Phytoseiulus macropilis* (Banks) to their prey *Tetranychus urticae* Koch (Poletti *et al.*, 2007). A repellent effect of thiacloprid has been found in the current study. The implication of this phenomenon for biological control of phytophagous mites is not yet fully understood.

The toxicity of neonicotinoids on predatory mites depends on species, strains, pesticide history, and experimental testing methods. This may explain the contrasting results reported for the most studied compounds. As an example, imidacloprid has been considered slightly toxic (Ahn *et al.*, 2004), moderately toxic (Duso *et al.*, 2008) or highly toxic towards *P. persimilis* (Blümel and Hausdorf, 2002; Sterk *et al.*, 2003). Present laboratory tests show that imidacloprid affected *K. aberrans* more than other neonicotinoids in terms of mortality. It also reduced egg production compared to acetamiprid. Bostanian *et al.*, (2009) also reported a higher toxicity of imidacloprid (and of acetamiprid) to *G. occidentalis* compared to thiamethoxam and thiacloprid. Villanueva and Walgenbach (2005) found similar trends when evaluating the mortality of *N. fallacis* adults exposed to leaf residues of different neonicotinoids. Bostanian *et al.*, (2010) conducted an additional evaluation on the toxicity of these neonicotinoids to *N. fallacis*. Imidacloprid and thiamethoxam were toxic to adults and reduced their fecundity; acetamiprid and thiacloprid showed lower toxicity levels and were recommended for field testing trials. Exposure to imidacloprid increased egg production of the predatory mite *Amblyseius victoriensis* (James, 1997) but this observation was not later confirmed. The repellent effect of some neonicotinoids requires additional studies. In our studies thiacloprid significantly affected *K. aberrans* escape rate, a parameter likely associated with repellence. Bostanian *et al.*, (2009) also observed repellent effects of thiacloprid on *G. occidentalis* in the laboratory. The implications of this phenomenon need to be investigated.

The compatibility of neonicotinoids with IPM is a matter of discussion. Imidacloprid and thiamethoxam have been considered good candidates for IPM on some crops (Lee *et al.*, 2002). However, Grafton-Cardwell *et al.*, (2008) and Bostanian *et al.*, (2009) considered imidacloprid and acetamiprid incompatible with IPM programmes. It has been argued that the response of spider mites to neonicotinoids application can be strain dependent (Ako *et al.*, 2006). Repeated applications of acetamiprid were associated with spider mite (*P. ulmi* and *T. urticae*) outbreaks in US apple orchards (Beers *et al.*, 2005). This situation was less frequent

with thiacloprid and clothianidin. According to these authors neonicotinoids did not eliminate predatory mites but they inhibited their response to increasing prey populations. Comparative toxicity studies may help in understanding the impact of a pesticide in realistic conditions. Stavrinides and Mills (2009) evaluated the impact of imidacloprid on the growth rate of the spider mite *Tetranychus pacificus* McGregor and its predatory mite *G. occidentalis*. Imidacloprid led *G. occidentalis* populations close to extinction, allowing *T. pacificus* populations to increase. They recommended evaluating the demographic effects of pesticides on pests and natural enemies for a full assessment of pesticide impacts on biological control. Another relevant topic is the route of exposure. Cloyd and Bethke (2011) reviewed the possible ways of contamination of predators by neonicotinoids (e.g. ingestion of prey, pollen, nectar, plant tissue or plant fluids contaminated by the pesticides) and stressed the potential importance of interactions between plants or flowers and pesticides (e.g. concentration and metabolites). In another study the effect of thiamethoxam on *T. urticae* and its predator *P. persimilis* was evaluated by considering different routes of exposure. Residual and contaminated food exposures caused higher effects than topical exposure on both mite species. Combinations of all routes of exposure caused effects higher than 90%. The impact of the pesticide was more favourable to *P. persimilis* than to its prey by limiting thiamethoxam exposure to ingestion of contaminated food (Pozzebon *et al.*, 2011).

In field trials *K. aberrans* populations were not affected by chlorpyrifos according to a recent evaluation on its resistance to organophosphates (Tirello *et al.*, 2012). However chlorpyrifos reduced predatory mite fecundity, confirming trends seen for OP resistant *Amblyseius andersoni* strains (Duso *et al.*, 1992). The compatibility of chlorpyrifos with IPM strategies aimed at enhancing the performance of *K. aberrans* depends on pesticide history (Mori *et al.*, 1999).

Lufenuron and methoxyfenozide showed low effects on *K. aberrans* strain survival with some effect on fecundity, while indoxacarb proved to be the most selective among the tested pesticides. Our results confirm the findings of some field trials conducted on *K. aberrans* (Mori *et al.*, 1999; Tosi *et al.*, 2006) or other predatory mite species (Rodrigues *et al.*, 2004).

The present study shed light on lethal and sub-lethal effects of pesticides frequently used in orchards. For most pesticides results were consistent with those of field trials available in the literature. This suggests that laboratory studies with *K. aberrans* can predict most effects induced by field applications of pesticides on this beneficial.

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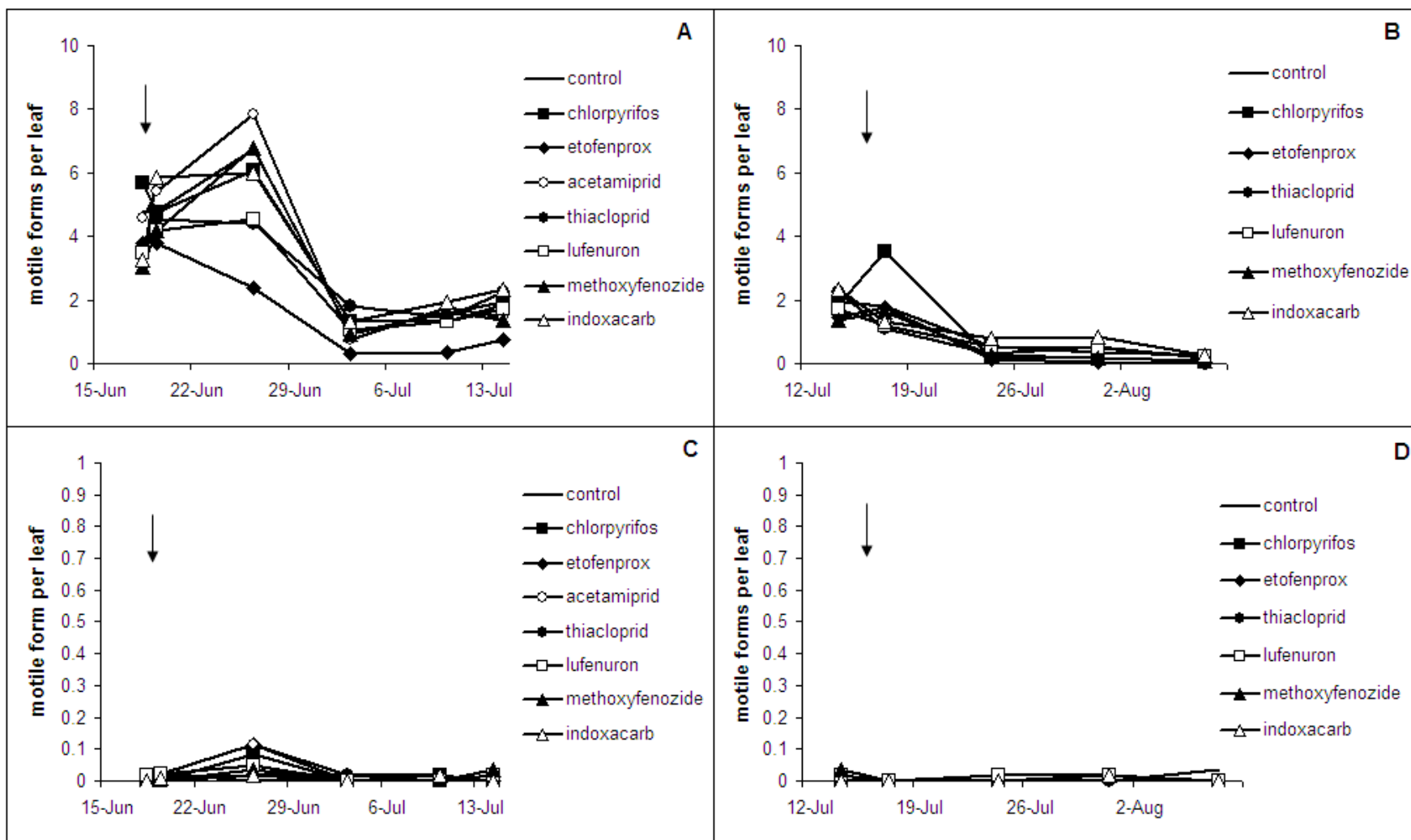


Figure 1 - Population dynamics of *Kampimodromus aberrans* (A-B) and *Panonychus ulmi* (C-D) observed in the field experiment during 2009. Arrows indicate insecticide application.

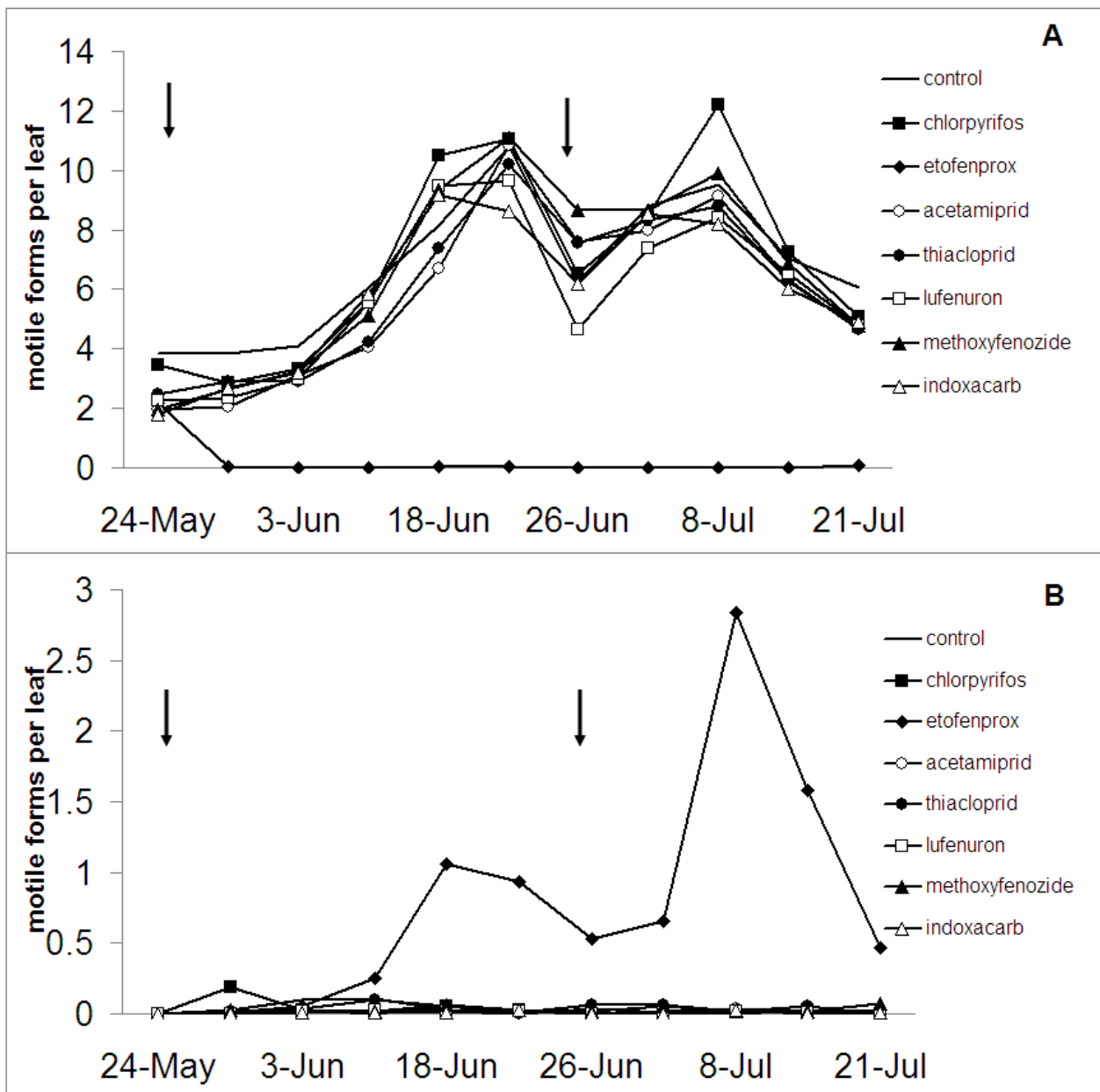


Figure 2 - Population dynamics of *Kampimodromus aberrans* (A) and *Panonychus ulmi* (B) observed in the field experiment during 2010. Arrows indicate insecticide application.

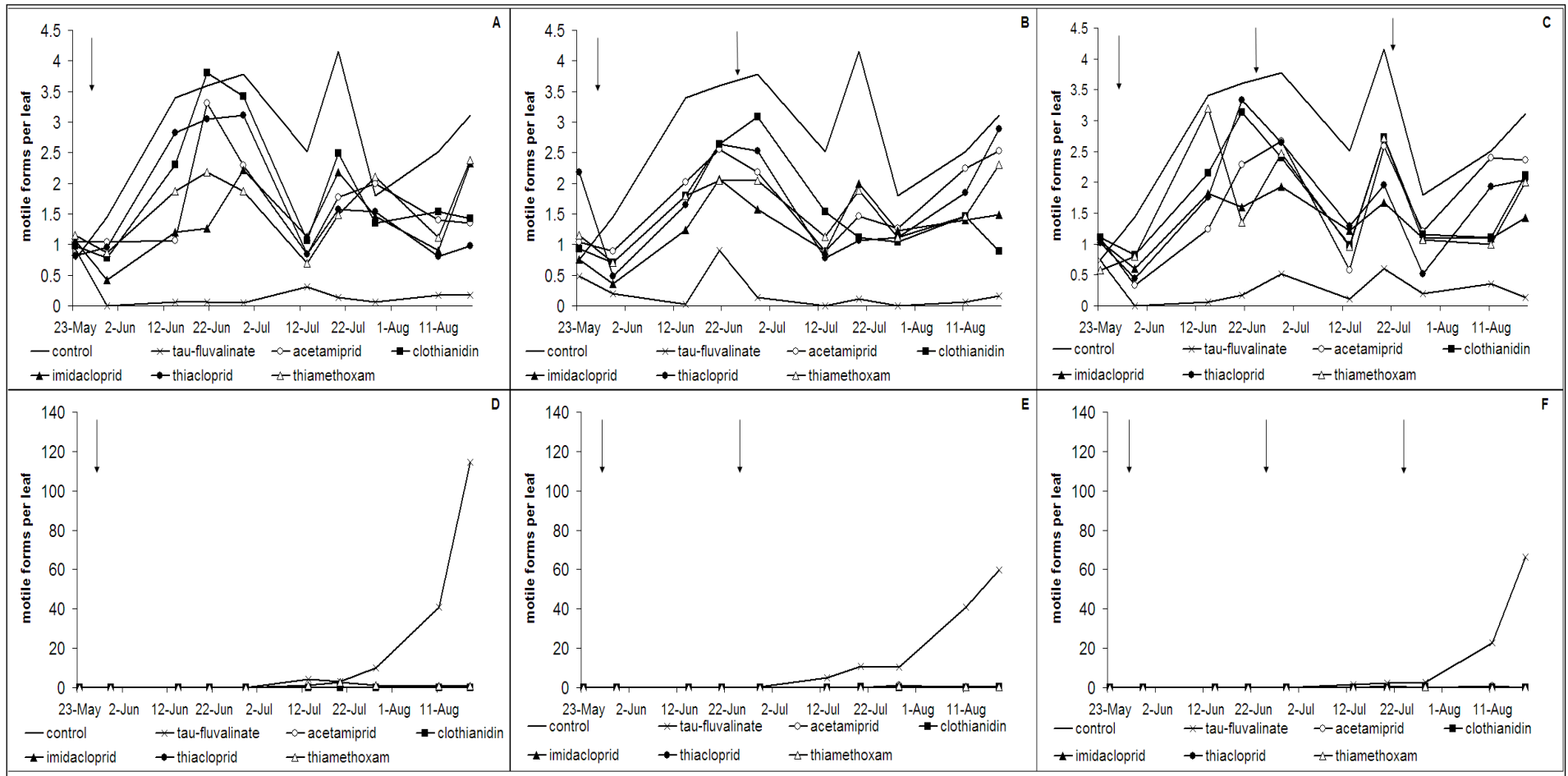


Figure 3 - Population dynamics of *Kampimodromus aberrans* (A, B, C) and *Panonychus ulmi* (D, E, F) observed in the first field experiment of 2011. Arrows indicate insecticide applications.

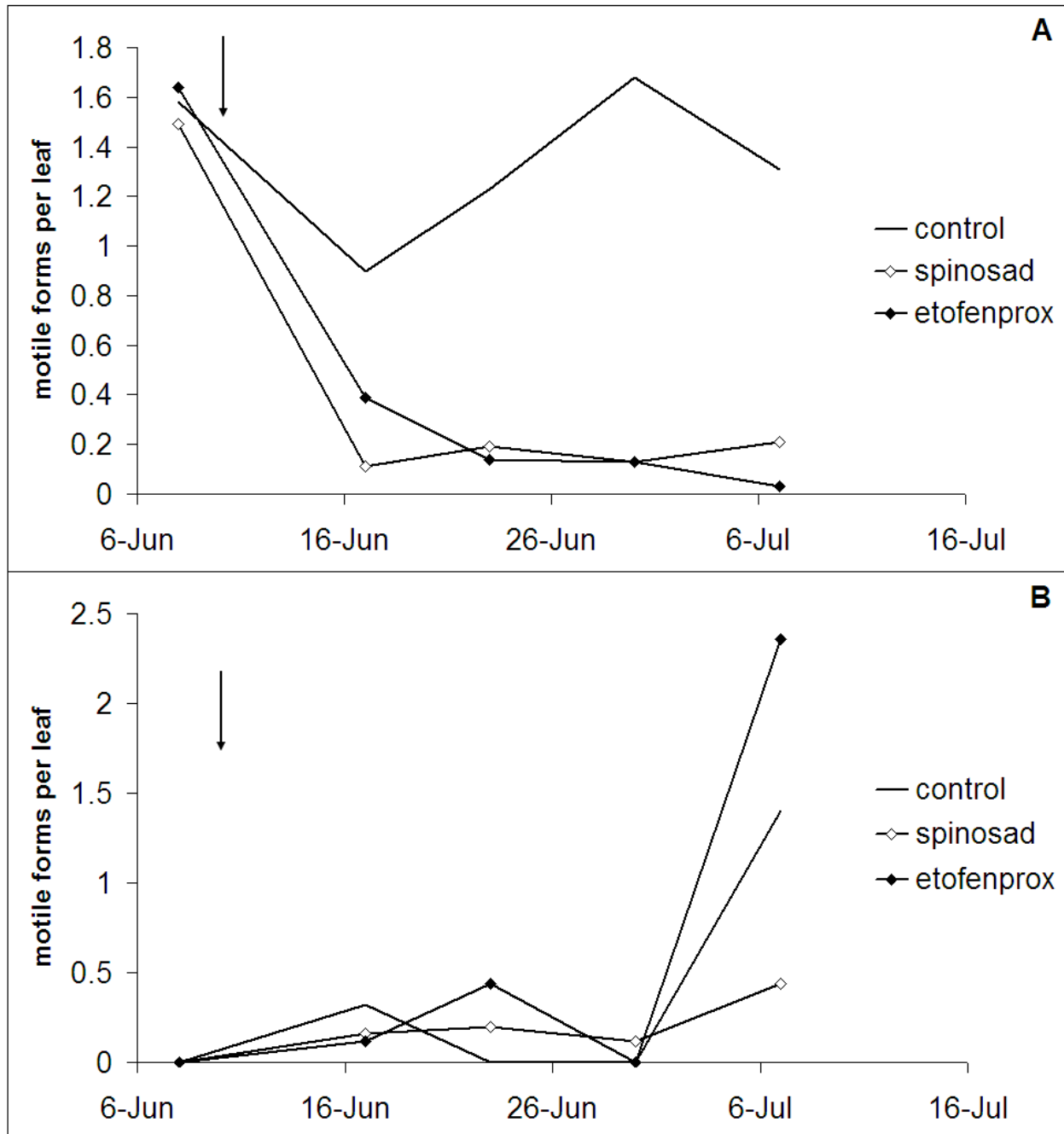


Figure 4 - Population dynamics of *Kampimodromus aberrans* (A) and *Panonychus ulmi* (B) observed in the second field experiment of 2011. Arrows indicate insecticide applications.



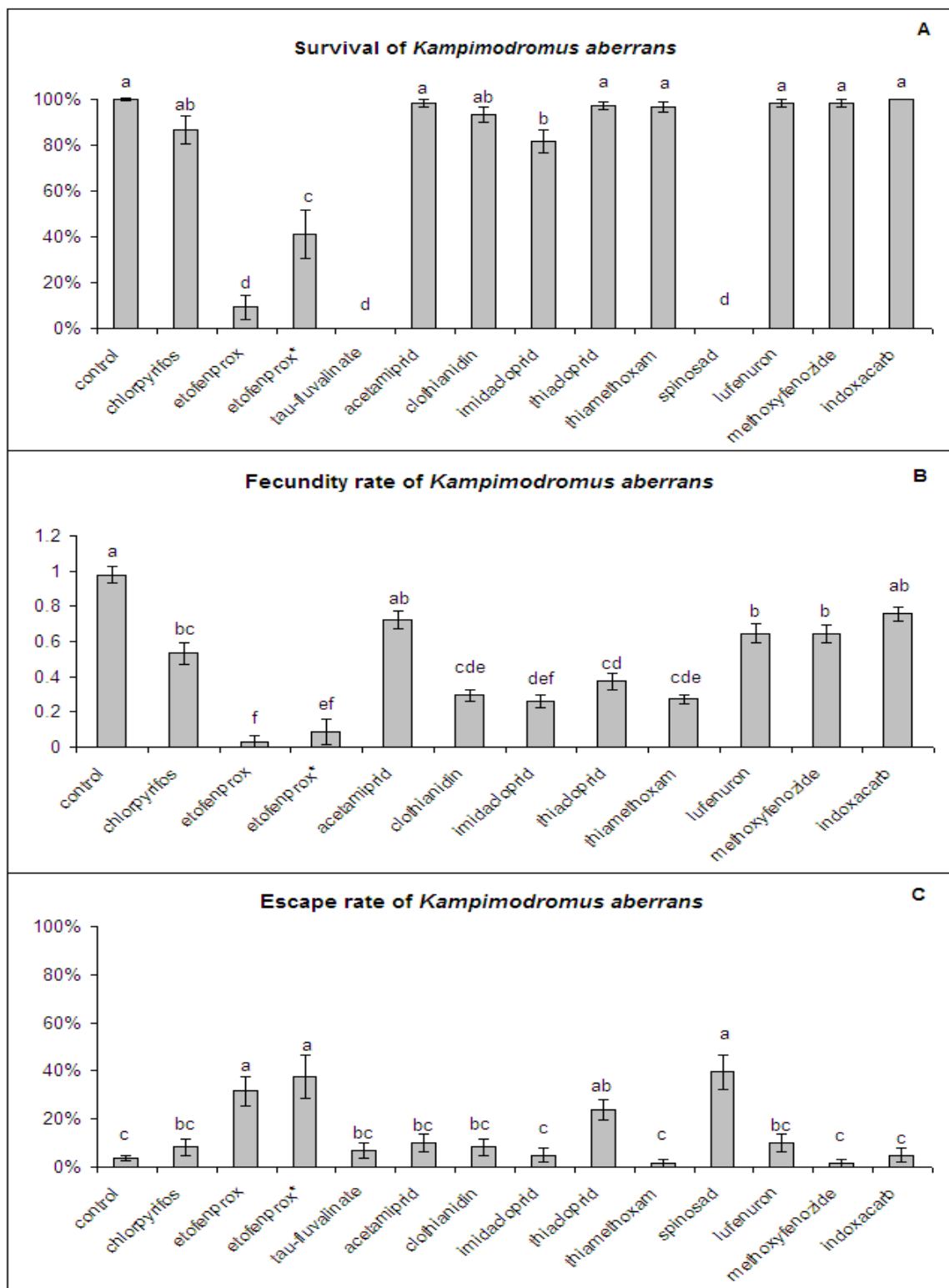


Figure 5 - Effects of selected insecticides on survival (A) fecundity (B) and escape rate (C) of *Kampimodromus aberrans* in laboratory tests. Etofenprox\* indicates treatment with the commercial formulation Trebon Up<sup>®</sup>.

**Table 1 - Pesticides applied in laboratory and field trials**

IRAC Group	Chemical group	Sub-	Active Ingredient	Trade Mark	Dose	Concentration of Formulation	Field
1B	Organophosphates		Chlorpyrifos	Dursban® 75WG	70 g hL <sup>-1</sup>	–	2009 2010
3A	Pyrethroids		Etofenprox	Trebon star®	100 ml hL <sup>-1</sup>	158 g L <sup>-1</sup>	2009 2010
				Trebon Up	100 ml hL <sup>-1</sup>	200 g L <sup>-1</sup>	2011
			Tau-fluvalinate	Klartan® 20EW	75 ml hL <sup>-1</sup>	240 g L <sup>-1</sup>	2011
4A	Neonicotinoids		Acetamiprid	Epik®	37.5 g hL <sup>-1</sup>	–	2009 2010 2011
			Clothianidin	Dantop® 50WG	15 g hL <sup>-1</sup>	–	2011
			Imidacloprid	Confidor® 200SL	50 ml hL <sup>-1</sup>	200 g L <sup>-1</sup>	2011
			Thiamethoxam	Actara® 25WG	30 g hL <sup>-1</sup>	–	2011
5	Spinosyns		Spinosad	Laser®	20 ml hL <sup>-1</sup>	480 g L <sup>-1</sup>	–
15	Benzoylureas		Lufenuron	Match Top®	100 ml hL <sup>-1</sup>	50 g L <sup>-1</sup>	2009 2010
18	Diacylhydrazines		Methoxyfenozide	Prodigy®	40 ml hL <sup>-1</sup>	240 g L <sup>-1</sup>	2009 2010
22A	Oxadiazine		Indoxacarb	Steward®	16.5 g hL <sup>-1</sup>	-	2009 2010

**Table 2** - Effects of selected pesticides on survival and fecundity of *K. aberrans*; the coefficient of toxicity is also reported. Etofenprox\* indicates treatment with the commercial formulation Trebon Up<sup>®</sup>.

<b>Treatments</b>	<b>Corrected mortality (%)</b>	<b>Corrected fecundity</b>	<b>E (%)</b>
Chlorpyrifos	13.33	0.54	52.86
Etofenprox	90.74	0.03	99.70
Etofenprox*	58.82	0.09	96.36
Tau-fluvalinate	100.00	0.00	100.00
Acetamiprid	1.67	0.74	27.59
Clothianidin	6.67	0.30	72.04
Imidacloprid	18.33	0.26	78.36
Thiacloprid	2.78	0.38	62.98
Thiamethoxam	3.33	0.28	73.03
Spinosad	100.00	0.00	100.00
Lufenuron	1.67	0.66	35.15
Methoxyfenozide	1.67	0.66	35.10
Indoxacarb	0.00	0.77	22.79

**Table 3** - Results of pairwise t-test on the least square means of *Kampimodromus aberrans* population observed in different treatments during 2011. Degree of freedom = 121. Bold numbers indicate significant differences after Bonferroni adjustment of the critical alpha value (0.05/121 = 0.00041).

active ingredient	number of applications	thiamethoxam			thiacloprid			imidacloprid			clothianidin			acetamiprid			tau-fluvalinate		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
control	t	<b>5.22</b>	<b>4.91</b>	<b>5.27</b>	<b>5.01</b>	<b>4.60</b>	<b>4.70</b>	<b>5.97</b>	<b>6.55</b>	<b>6.09</b>	<b>3.64</b>	<b>5.37</b>	<b>4.25</b>	<b>4.84</b>	<b>4.29</b>	<b>4.89</b>	<b>16.82</b>	<b>16.76</b>	<b>15.84</b>
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
thiamethoxam	1	t	-0.31	0.05	-0.21	-0.62	-0.51	0.75	1.33	0.87	-1.58	0.15	-0.96	-0.38	-0.93	-0.33	<b>11.60</b>	<b>11.54</b>	<b>10.62</b>
		P	0.7569	0.9567	0.8323	0.5347	0.6086	0.4556	0.1869	0.3833	0.1175	0.8831	0.3367	0.703	0.3565	0.7422	<0.0001	<0.0001	<0.0001
	2	t		0.36	0.10	-0.31	-0.20	1.06	1.64	1.19	-1.27	0.46	-0.65	-0.07	-0.62	-0.02	<b>11.91</b>	<b>11.85</b>	<b>10.93</b>
		P		0.716	0.9221	0.7553	0.8393	0.2918	0.1041	0.2383	0.2079	0.648	0.5142	0.9428	0.5394	0.9845	<0.0001	<0.0001	<0.0001
	3	t			-0.27	-0.68	-0.57	0.69	1.27	0.82	-1.63	0.09	-1.02	-0.44	-0.98	-0.38	<b>11.55</b>	<b>11.49</b>	<b>10.56</b>
		P			0.7902	0.4997	0.5712	0.489	0.2055	0.4135	0.1055	0.926	0.3103	0.6632	0.329	0.7016	<0.0001	<0.0001	<0.0001
thiacloprid	1	t				-0.41	-0.30	0.96	1.54	1.09	-1.36	0.36	-0.75	-0.17	-0.71	-0.12	<b>11.81</b>	<b>11.76</b>	<b>10.83</b>
		P				0.6822	0.7637	0.3386	0.1263	0.2791	0.175	0.7197	0.4534	0.8653	0.4769	0.9067	<0.0001	<0.0001	<0.0001
	2	t					0.11	1.37	1.95	1.5	-0.95	0.77	-0.34	0.24	-0.3	0.29	<b>12.22</b>	<b>12.17</b>	<b>11.24</b>
		P					0.9133	0.1729	0.0535	0.1368	0.3421	0.4428	0.7331	0.8104	0.7624	0.7701	<0.0001	<0.0001	<0.0001
	3	t						1.26	1.84	1.39	-1.06	0.66	-0.45	0.13	-0.41	0.18	<b>12.11</b>	<b>12.06</b>	<b>11.13</b>
		P						0.2094	0.0681	0.1676	0.2899	0.5099	0.6528	0.8958	0.6809	0.8545	<0.0001	<0.0001	<0.0001
imidacloprid	1	t							0.58	0.13	-2.32	-0.60	-1.71	-1.13	-1.67	-1.08	<b>10.85</b>	<b>10.79</b>	<b>9.87</b>
		P							0.5637	0.8995	0.0217	0.5489	0.0893	0.2604	0.0967	0.2831	<0.0001	<0.0001	<0.0001
	2	t								-0.45	-2.90	-1.18	-2.29	-1.71	-2.25	-1.66	<b>10.27</b>	<b>10.22</b>	<b>9.29</b>
		P								0.6518	0.0044	0.2403	0.0236	0.0899	0.0261	0.1001	<0.0001	<0.0001	<0.0001
	3	t									-2.45	-0.73	-1.84	-1.26	-1.80	-1.20	<b>10.72</b>	<b>10.67</b>	<b>9.74</b>
		P									0.0157	0.4683	0.0683	0.2111	0.0743	0.2307	<0.0001	<0.0001	<0.0001
clothianidin	1	t									1.72	0.61	1.19	0.65	1.25	<b>13.18</b>	<b>13.12</b>	<b>12.20</b>	
		P									0.0873	0.5417	0.2347	0.5164	0.2149	<0.0001	<0.0001	<0.0001	
	2	t										-1.11	-0.53	-1.07	-0.48	<b>11.45</b>	<b>11.40</b>	<b>10.47</b>	
		P										0.2684	0.5974	0.2854	0.6342	<0.0001	<0.0001	<0.0001	
	3	t											0.58	0.04	0.63	<b>12.56</b>	<b>12.51</b>	<b>11.58</b>	
		P											0.5615	0.9691	0.5268	<0.0001	<0.0001	<0.0001	
acetamiprid	1	t												-0.54	0.05	<b>11.98</b>	<b>11.93</b>	<b>11.00</b>	
		P												0.5878	0.9582	<0.0001	<0.0001	<0.0001	
	2	t													0.6	<b>12.53</b>	<b>12.47</b>	<b>11.54</b>	
		P													0.5523	<0.0001	<0.0001	<0.0001	
	3	t														<b>11.93</b>	<b>11.87</b>	<b>10.95</b>	
		P														<0.0001	<0.0001	<0.0001	
tau-fluvalinate	1	t															-0.06	-0.98	
	P																0.9548	0.3287	
2	t																	-0.92	
P																		0.3574	

**Table 4** - Results of pairwise t-test on the least square means of *Panonychus ulmi* population observed in different treatments during 2011. Degree of freedom = 63.9. Bold numbers indicate significant differences after Bonferroni adjustment of the critical alpha value ( $0.05/63.9 = 0.00078$ ).

active ingredient	number of applications	thiamethoxam			thiacloprid			imidacloprid			clothianidin			acetamiprid			tau-fluvalinate		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
control	t	2.03	0.58	0.40	0.37	0.03	0.08	0.03	0.16	0.00	0.05	-0.28	-0.24	-0.19	-0.59	-0.63	<b>-9.00</b>	<b>-10.56</b>	<b>-8.09</b>
	P	0.0468	0.5615	0.6935	0.7154	0.976	0.9352	0.9745	0.8734	0.9985	0.9614	0.783	0.8136	0.8502	0.5562	0.5306	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
1	t		1.44	1.63	1.66	2.00	1.95	2.00	1.87	2.03	2.08	1.75	1.79	1.84	1.44	1.40	<b>-6.97</b>	<b>-8.53</b>	<b>-6.07</b>
	P		0.1537	0.1077	0.1016	0.0501	0.0561	0.0503	0.0664	0.0466	0.0419	0.0848	0.0781	0.0707	0.1559	0.1673	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
thiamethoxam	2			0.19	0.22	0.55	0.50	0.55	0.42	0.59	0.63	0.31	0.35	0.39	-0.01	-0.05	<b>-8.41</b>	<b>-9.98</b>	<b>-7.51</b>
	P			0.8517	0.8286	0.5819	0.6174	0.5832	0.6733	0.5603	0.5295	0.7598	0.7299	0.6949	0.9937	0.9627	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
3	t				0.03	0.37	0.31	0.36	0.24	0.4	0.44	0.12	0.16	0.21	-0.2	-0.23	<b>-8.60</b>	<b>-10.17</b>	<b>-7.7</b>
	P				0.9764	0.7158	0.7544	0.7172	0.8143	0.6921	0.6582	0.9054	0.8741	0.8373	0.8455	0.8152	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
1	t					0.34	0.28	0.33	0.21	0.37	0.41	0.09	0.13	0.18	-0.23	-0.26	<b>-8.63</b>	<b>-10.20</b>	<b>-7.73</b>
	P					0.7379	0.7769	0.7394	0.8373	0.714	0.6796	0.9288	0.8974	0.8604	0.8224	0.7924	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
thiacloprid	2						-0.05	0.00	-0.13	0.03	0.08	-0.25	-0.21	-0.16	-0.56	-0.60	<b>-8.97</b>	<b>-10.53</b>	<b>-8.06</b>
	P						0.9591	0.9985	0.8971	0.9745	0.9374	0.8062	0.837	0.8738	0.5765	0.5504	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
3	t							0.05	-0.08	0.08	0.13	-0.19	-0.16	-0.11	-0.51	-0.55	<b>-8.91</b>	<b>-10.48</b>	<b>-8.01</b>
	P							0.9606	0.9378	0.9337	0.8968	0.8461	0.8772	0.9143	0.6118	0.585	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
1	t								-0.13	0.03	0.08	-0.24	-0.20	-0.16	-0.56	-0.60	<b>-8.96</b>	<b>-10.53</b>	<b>-8.06</b>
	P								0.8986	0.973	0.9359	0.8076	0.8384	0.8753	0.5778	0.5516	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
imidacloprid	2								0.16	0.21	-0.12	-0.08	-0.03	-0.43	-0.47		<b>-8.84</b>	<b>-10.40</b>	<b>-7.93</b>
	P								0.8719	0.8354	0.9076	0.939	0.9764	0.6675	0.6396		<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
3	t									0.05	-0.28	-0.24	-0.19	-0.59	-0.63		<b>-9.00</b>	<b>-10.56</b>	<b>-8.10</b>
	P									0.9629	0.7815	0.8121	0.8487	0.5549	0.5294		<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
1	t											-0.33	-0.29	-0.24	-0.64	-0.68	<b>-9.04</b>	<b>-10.61</b>	<b>-8.14</b>
	P											0.7461	0.7762	0.8124	0.5243	0.4995	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
clothianidin	2											0.04	0.09	-0.32	-0.35		<b>-8.72</b>	<b>-10.29</b>	<b>-7.82</b>
	P											0.9684	0.931	0.7538	0.7245		<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
3	t													0.05	-0.35	-0.39	<b>-8.76</b>	<b>-10.33</b>	<b>-7.86</b>
	P													0.9626	0.7239	0.6951	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
1	t														-0.40	-0.44	<b>-8.81</b>	<b>-10.37</b>	<b>-7.90</b>
	P														0.6891	0.6608	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
acetamiprid	2														-0.04		<b>-8.40</b>	<b>-9.97</b>	<b>-7.50</b>
	P														0.969		<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
3	t																<b>-8.37</b>	<b>-9.93</b>	<b>-7.46</b>
	P																<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
tau-fluvalinate	1																	-1.57	0.90
	P																	0.1223	0.3705
2	t																		2.47
	P																		

## **Chapter V**

### **Does pollen availability mitigate the impact of pesticides on predatory mites?**

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The manuscript in preparation as:

Alberto Pozzebon, Shakeel Ahmad, Paola Tirello, Mauro Lorenzon and Carlo Duso - Does pollen availability mitigate the impact of pesticides on predatory mites?

In this work, I collected most of the data, contributed to the analysis and drafted the manuscript

## Abstract

The enhancement of biological control tactics for a reduction in pesticide use in agriculture is an important issue in agro-ecosystem management studies. In the last decades, there has been increasing interest in conservation biological control, which includes agricultural practices aimed at the promotion of natural enemies through the provision of plant-derived food sources for natural enemies. In several agricultural systems, natural enemies are endangered by the insecticide necessary to control key pests. Here we investigate the impact of the provision of plant-derived food sources on the effects of pesticides on beneficial predatory mites.

Experiments were performed using *Kampimodromus aberrans*, which is a generalist predatory mite of phytophagous mites on several perennial crops. In laboratory we evaluated the influence of pollen dose and pollen application frequency on lethal and sub-lethal effect of two pesticides (chlorpyrifos, spinosad). Using potted plant experiment, the effects of pesticides and pollen were assessed at predatory mite population level.

Predatory mite fecundity increased by augmenting pollen amount and application frequency. Survival and fecundity were reduced by insecticides: spinosad was more toxic than chlorpyrifos. High pollen application frequency compensated for the negative effect of chlorpyrifos. Potted plant experiment confirms also at predatory mite population level the positive effect of pollen application and the negative effect of insecticide treatments. Spinosad was confirmed to be detrimental, independently from pollen application. Pollen application reduced the impact of chlorpyrifos on *K. aberrans*. Without pollen application predatory mites numbers were similar between spinosad and chlorpyrifos.

Results obtained here highlighted the importance of the nutritional quality of food provided to predatory mites. The provision of fresh pollen is of particular importance when pesticides are applied. Pollen can be contaminated by insecticides and the provision of uncontaminated fresh pollen can decrease the exposure to pesticides with a reduction in their detrimental effects. The findings obtained here stress that management practices aimed at food source availability can mitigate the effect of pesticides on natural enemies, promoting their persistence in agro-ecosystems.

## Introduction

The enhancement of biological control tactics for a reduction in pesticide use in agriculture is an important issue in agro-ecosystem management studies (Bale *et al.*, 2008; Pretty, 2008; Whittingham 2011) and it is also considered in European policy (e.g., Directive 2009/128/EC). In the last decades, there has been increasing interest in conservation biological control, which includes agricultural practices aimed at modifying the environment for the enhancement and protection of specific natural enemies (Barbosa 1998; Landis *et al.*, 2000; Jonsson *et al.* 2008; Gurr *et al.*, 2004). Conservation biological control tactics are mainly based on the promotion of natural enemies characterized by a certain degree of polyphagy (Symondson *et al.*, 2002), and an important aspect of this is the provision of plant-derived food sources for natural enemies (Coll and Guershon, 2002; Wäckers *et al.*, 2005). Plant-provided food can improve survival, longevity and fecundity of beneficial arthropods, and is of particular importance to overcome periods of prey shortage (Wäckers, 2005; Lundgren, 2009). Several measures have been studied to optimize plant-derived food sources, such as the provision of food-providing plants and food spray (Landis *et al.*, 2000; Wade *et al.*, 2008; Simpson *et al.*, 2011b).

In several agricultural systems the major limitations for natural enemies are the insecticide treatments necessary to control key pests. The detrimental effects of pesticides on the complex of natural enemies can induce secondary pest outbreaks (e.g., Croft 1990; Ruberson *et al.*, 1998) with a consequent increase in pesticides use (Steinmann *et al.*, 2011). A reduction of the adverse effects of pesticides is a prerequisite for other conservation practices (Landis *et al.*, 2000). Several researches have investigated pesticide selectivity on beneficial non-target arthropods with the purpose of minimizing the risk associated with pesticide use (e.g., Hassan *et al.*, 1994; Sterk *et al.*, 1999). Among conservation practices, some studies evidenced that an increase of refuge habitats can help in maintaining the presence of predators in insecticide treated fields (Lester *et al.*, 1998; Lee *et al.*, 2001). However, there is a lack of knowledge on the impact of the provision of plant-derived food sources in crop systems where pesticides are extensively used.

In this paper we investigated this aspect considering the consequences of plant-derived food provision on the impact of insecticide treatments on generalist predatory mites. We focused on pollen, which is an extremely rich source of nutrients essential for the development and reproduction of various biological control agents (Lundgren, 2009). It is of particular importance as an alternative food source to prey for generalist predatory mites



(Acari: Phytoseiidae) (McMurtry and Croft, 1997). These predators play a key role in controlling spider mites and eriophyoids on perennial crops (e.g., Sabelis 1985; Duso *et al.*, 2010) and are also bio-indicators for pesticide impact on terrestrial arthropod communities (Candolfi *et al.*, 1999; Beaulieu and Weeks, 2007; Bernard *et al.*, 2010). Field observations showed that phytoseiid population dynamics on plants are often correlated to pollen availability on leaves suggesting that they can persist by exploiting pollen in the absence of prey (e.g., McMurtry and Johnson, 1965; Eichhorn and Hoos, 1990; van Rijn and Tanigoshi, 1999a; Addison *et al.*, 2000; Duso *et al.*, 2004). In cultivated plant systems, pollen availability promote increase in generalist predatory mite population levels (e.g., Kennett, *et al.*, 1979; Engel and Ohnesorge, 1994b; Aguilar-Fenollosa *et al.*, 2011; Montserrat *et al.*, 2011), and can lead to biocontrol improvement (Nomikou *et al.*, 2002; Van Rijn *et al.*, 2002; Onzo *et al.*, 2005; Gonzalez-Fernandez *et al.*, 2009; Nomikou *et al.*, 2010; Maoz *et al.*, 2011). It is well known that pollen can have a positive effect on oviposition, survival and longevity of predatory mites, in some cases resulting as higher or comparable to those of prey (Duso & Camporese 1991; Schausberger 1992; Engel & Ohnesorge 1994a; Wei & Walde 1997; Vantornhout *et al.* 2004; Ragusa, Tsolakis & Palomero 2009; van Maanen *et al.* 2010) or other non-prey food substances (Tanigoshi *et al.*, 1993; van Rijn and Tanigoshi, 1999b; Gnanvossou *et al.*, 2005; Pozzebon and Duso, 2008; Pozzebon *et al.*, 2009). On the other hand, life history parameters of beneficials are negatively influenced by pesticides (Stark *et al.*, 2004; Desneux *et al.*, 2007; Stark *et al.*, 2007). The response by generalist predatory mites in terms of life history is a topic of basic research in the evaluation of pesticide risks for non-target arthropods (Bakker 1995; Candolfi *et al.*, 2000 and 2001). Here we evaluated the effect of pollen provision on the impact of pesticides on predatory mites at individual and population level. The effect of experimental factors was evaluated on life-history parameters in laboratory experiments and the effect of pollen and insecticides application were evaluated on predatory mite population in a plant-scale manipulative experiment.

## Materials and methods

### Study system

Experiments were performed using *Kampimodromus aberrans* (Oudemans), which is a generalist predatory mite inhabiting perennial crops (Chant 1959; Espinha *et al.* 1995; Kasap 2005; Miñarro *et al.*, 2005; Broufas *et al.*, 2007). Its role as biocontrol agent has been investigated on grapevines (e.g., Duso 1989; Kreiter *et al.*, 2000; Tixier *et al.*, 2002; Duso and Vettorazzo, 1999), hazelnuts (e.g., Nicotina and Cioffi 1998; Tsolakis *et al.*, 2000; Ozman-Sullivan, 2006), and apple orchards (El-Borolossy and Fischer-Colbrie 1989; Schausberger, 1992; Duso *et al.*, 2009). This study was performed using a *K. aberrans* strain proved to be resistant to organophosphate insecticides (Tirello *et al.*, 2012) collected from a commercial apple orchard located at San Michele all'Adige (Trento province, Trentino-Alto Adige Region). Prior to the experiments, predatory mite colonies were maintained on mass rearing units using various *taxa* pollen as food.

We used pollen of cattail *Typha latifolia* L. in the experiments because is a naturally occurring pollen in several agricultural areas, it is easy to collect and has often been used as standard food for predatory mite rearing (Van Rijn and Tanigoshi, 1999; Roda *et al.*, 2001; Pozzebon., 2009; Park *et al.*, 2011). Pollen was collected during summer and stored in a freezer at -20 °C until use.

Among pesticides used in apple orchards against the codling moth *Cydia pomonella* L. we selected spinosad (Laser ®, Dow AgroSciences, 14.4 a.i. g/hl) and chlorpyrifos (Dursban ®, Dow AgroSciences, 31.31 a.i. g/hl).

### Laboratory experiments

In the first experiment we assessed the suitability of cattail pollen as food source for *K. aberrans* as compared to mite prey. The life table parameters of predatory mites feeding on pollen were compared with those feeding on the spider mite *Panonychus ulmi* (Koch). Sixty one-day-old eggs were transferred singly to experimental units consisting of apple leaf discs placed on a wet layer of cotton in a plastic box. They were maintained at controlled conditions ( $23 \pm 1$  °C,  $70 \pm 10\%$  R.H., 16L: 8D photoperiod) until the end of the experiments. *Panonychus ulmi* juvenile stages or cattail pollen were provided daily. The duration of all developmental stages (egg, larva, protonymph, deutonymph, adult) and their survival rate on both diets were recorded every 12 hours. When adults were obtained, one male and one

female reared on the same food source were placed together in the same experimental unit provided with *P. ulmi* or pollen. Mite oviposition was also monitored every 24 hours. Oviposition was recorded until the females' death. Freshly laid eggs or larvae were regularly counted and transferred to a separate detached apple leaf rearing box to check the effects of food sources on sex ratio of phytoseiids. Life table parameters [Reproductive rate ( $R_0$ ); mean generation time ( $T$ ); intrinsic rate of increase ( $r_m$ ); doubling time ( $Dt$ ); finite rate of increase ( $\lambda$ )] were estimated and compared using a SAS-based procedure developed by Maia *et al.* (2000).

In another experiment predatory mites were exposed to fresh pesticide residues. In this experiment pollen was provided at two doses and two frequencies in order to assess its effect on the impact of pesticides on predatory mites at individual level. A factorial design was used and experimental factors were: insecticide application (chlorpyrifos, spinosad or untreated control), pollen dose (low: 0.03 mg/cm<sup>2</sup>; high: 0.33 mg/cm<sup>2</sup>) and pollen application frequency (low: once at the start of the experiment; high: every 48 h after the first 72 h). Apple leaf sections (6 cm<sup>2</sup>) were immersed in the pesticide solution for 30 sec (water was used in control treatments). When pesticide residues had dried, the leaf sections were used to form experimental units similar to those described above for rearing. Two coeval *K. aberrans* females were transferred onto leaf sections for a total of 40 females per treatment. Prior to females transfer, pollen was provided according to the experimental protocol. Experimental arenas were maintained in a climatic chamber at 25 ± 2 °C, 60 ± 10 % RH and a photoperiod of 16L: 8D. Female survival and escapes were recorded at 72 and 168 h after treatments and fecundity was recorded for 168 h. Data on survival and escapes were analyzed using a factorial logistic regression with GENMOD procedure of SAS (SAS Institute, 1999) and a likelihood ratio G test ( $\alpha = 0.05$ ) to evaluate effect of experimental factors and their interaction. Differences among treatments were evaluated with a Wald chi-square test ( $\alpha = 0.05$ ) to the least-square means. Data on fecundity were analyzed with a REML (Restricted Maximum Likelihood) model and the F test ( $p = 0.05$ ) was applied to assess experimental factors and their interaction effects. Differences among treatments were evaluated with a t-test ( $\alpha = 0.05$ ) to the least-square means. Prior to the analysis, data on fecundity were checked for normality and homoscedasticity and then transformed in log (x +1).

### **Potted plant experiment**

Pesticide and pollen augmentation effects were assessed at population level on potted apple plants colonized by *K. aberrans*. A factorial experimental design was used, where

experimental factors were: insecticide application (chlorpyrifos, spinosad and untreated control) and pollen application (pollen vs. no pollen). Each treatment comprised four plants with 7-10 shoots each. Predatory mites were released on 2-3 homogenous shoots (about 100 females per shoot) per plant seven days before insecticide applications using shoots collected from an apple orchard. Cattail pollen was sprayed onto selected shoots according to the experimental design. A strip of plumber's tape with a sticky barrier was placed at the base of each shoot to avoid mite escapes. Cattail pollen was provided on a half of these plants every two days using an experimental sprinkler (Girolami *et al.* 2000; Baldessari 2005). Mite densities and their developmental stages were assessed prior to insecticide applications and thereafter at 3, 7 and 15 days after treatments by removing five leaves per shoot. Collected leaves were analyzed in the laboratory under a dissecting microscope.

Data on population density were analyzed with a Restricted Maximum Likelihood (REML) repeated measures model with the SAS MIXED procedure (SAS Institute Inc., 1999). Densities of *K. aberrans* adults (male and adults), juveniles (protonymphs, deutonymphs and eggs) and motile forms (adults and juveniles), were analyzed separately and considered as response variables with repeated measures made at different times, i.e. sampling dates. Plant was considered as random effect term. Using an F test ( $\alpha = 0.05$ ) we evaluated the effect of experimental factors, time and their interactions. Slice option was used to partition F test of interactions between insecticide application and time. Interaction contrasts were designed with the ESTIMATE statement and tested using a t-test ( $\alpha = 0.05$ ). Moreover differences among treatments at each sampling date were evaluated using a t-test to the least-square means ( $\alpha = 0.05$ ). The Kenward-Roger method was used for degrees of freedom estimation (Littell *et al.*, 1996). According to Aikake's Information Criterion, first-order autoregressive proved to be the best fitting covariance structure for correlating different sampling dates (Littell *et al.*, 1996). Data were checked for analysis assumptions and untransformed data were used.

## Results

### Laboratory experiments

#### Effect of pollen on life-table parameters of *K. aberrans*

*Panonychus ulmi* and cattail pollen were suitable food sources for the development of *K. aberrans*. Food sources affected some life-table parameters of *K. aberrans*: feeding on pollen resulted in higher  $r_m$ ,  $R_0$ ,  $\lambda$ , and lower  $Dt$  than feeding on *P. ulmi*. Mean generation time ( $T$ ) was not influenced by food type (Table 1).

#### Effect of pesticides and pollen on predatory mites survival and fecundity

Laboratory studies evidenced a significant effect of insecticide applications on *K. aberrans* survival observed 72 h after treatments ( $G = 352.87$ ;  $df = 2$ ;  $p < 0.001$ ). Survival was higher in the control than in chlorpyrifos and spinosad treatments (Figure 1). Survival was not affected by different pollen amounts ( $G = 0.89$ ;  $df = 1$ ;  $p = 0.346$ ; Figure 2). Insecticides affected escape rate when assessed at 72 h ( $G = 70$ ;  $df = 2$ ;  $p < 0.001$ ), which resulted as higher in spinosad than in other treatments (Figure 4). Pollen amount did not affect escape rate assessed at 72 h ( $G = 0.39$ ;  $df = 1$ ;  $p = 0.532$ ; Figure 5).

Insecticides influenced survival rate calculated at 168 h ( $G = 6.89$ ;  $df = 2$ ;  $p = 0.008$ ). Predatory mites did not survive spinosad, and significant mortality was induced by chlorpyrifos (Figure 1). No effects of pollen amount, nor pollen application frequency were observed on survival assessed at 168 h ( $G = 2.60$ ;  $df = 1$ ;  $p = 0.107$ ;  $G = 0.01$ ;  $df = 1$ ;  $p = 0.99$ ; respectively; Figures 2 - 3). At 168 h, insecticides affected escape rate ( $G = 33.37$ ;  $df = 2$ ;  $p < 0.001$ ) resulting higher where spinosad was applied compared to the control and chlorpyrifos treatments (Figure 4). No effects of pollen amount or pollen application frequency were observed on escape rate at 168 h ( $G = 0.01$ ;  $df = 1$ ;  $p = 0.987$ ;  $G = 0.71$ ;  $df = 1$ ;  $p = 0.40$ ; respectively; Figures 5 - 6).

Fecundity of *K. aberrans* was influenced by insecticide application ( $F = 19.72$ ;  $df = 1, 132$ ;  $p < 0.001$ ). No eggs were laid where spinosad was applied, and a higher fecundity was observed in the control than on chlorpyrifos (Figure 7). Predatory mite fecundity increased by augmenting pollen amount ( $F = 15.83$ ;  $df = 1, 132$ ;  $p < 0.001$ ; Figure 7) and application frequency ( $F = 17.58$ ;  $df = 1, 132$ ;  $p < 0.001$ ; Figure 7). A significant interaction “insecticide application\*pollen application frequency” was observed ( $F = 9.30$ ;  $df = 1, 132$ ;  $p = 0.002$ ). At

low pollen application frequency fecundity was higher in the control than on chlorpyrifos, while no effect of insecticides was observed at high pollen application frequency (Figure 7).

## **Potted plant experiment**

### **Pesticides, pollen and predatory mites: Population density**

A general effect of insecticide applications was observed on *K. aberrans* population density and this effect varied over time (Table 2). No differences among treatments were found prior to insecticide application ( $F_{1, 37.6} = 0.31$ ;  $P = 0.738$ ). After insecticides application motile forms were observed at higher level in control compared to chlorpyrifos ( $t_{20.5} = 4.01$ ;  $P = 0.0007$ ; Figure 8) and spinosad plots ( $t_{20.5} = 8.19$ ;  $P < 0.0001$ ; Figure 8). During the same period, densities of motile forms of predatory mites were higher on chlorpyrifos than spinosad ( $t_{20.5} = 4.18$ ;  $P < 0.0001$ ; Figure 8). The abundance of *K. aberrans* motile forms was also influenced by pollen applications (Table 2), resulting highest where pollen was provided (Figure 8). A significant interaction was found among insecticides, pollen application and time (Table 2). The interaction between pollen application and insecticides was not significant prior to insecticide applications ( $F_{1, 37.6} = 0.37$ ;  $P = 0.863$ ) but emerged thereafter. In particular, pollen application determined higher population levels in control ( $t_{20.5} = 3.34$ ;  $P = 0.003$ ; Figure 8) and chlorpyrifos ( $t_{20.5} = 4.39$ ;  $P = 0.0003$ ; Figure 8), but not in spinosad plots ( $t_{20.5} = 1.41$ ;  $P = 0.173$ ; Figure 8). Consequently, differences among insecticide treatments were influenced by pollen application. After insecticide application negative effects of chlorpyrifos (vs. control:  $t_{20.5} = 2.31$ ;  $P = 0.031$ ; Figure 8) and spinosad were found (vs. control:  $t_{20.5} = 6.75$ ;  $P < 0.031$ ; Figure 8) but motile forms were higher on chlorpyrifos than on spinosad where pollen was provided (vs. control:  $t_{20.5} = 2.31$ ;  $P = 0.031$ ; Figure 8). Without pollen application, motile form density was higher in the control compared to chlorpyrifos ( $t_{20.5} = 3.36$ ;  $P = 0.003$ ; Figure 8) and spinosad ( $t_{20.5} = 4.83$ ;  $P < 0.0001$ ; Figure 8), but there were no differences between insecticide treatments ( $t_{20.5} = 1.41$ ;  $P = 0.174$ ; Figure 8).

The effect of experimental factors on *K. aberrans* adults reflected those on motile forms (Table 2). The interaction among insecticides, pollen applications and time was significant (Table 2). This interaction was not significant prior to insecticide applications ( $F_{1, 37.1} = 0.51$ ;  $P = 0.769$ ). Considering the period after treatment a positive effect of pollen was found in control ( $t_{20.6} = 2.99$ ;  $P = 0.007$ ; Figure 8) and chlorpyrifos ( $t_{20.6} = 3.87$ ;  $P < 0.001$ ; Figure 8), but not in spinosad plots ( $t_{20.6} = 1.26$ ;  $P = 0.223$ ; Figure 8). In pollen application treatments, negative effects of chlorpyrifos (vs. control:  $t_{20.6} = 2.32$ ;  $P = 0.031$ ; Figure 8) and spinosad

were found (vs. control:  $t_{20,6} = 6.18$ ;  $P < 0.0001$ ; Figure 8). *K. aberrans* adults reached higher numbers in chlorpyrifos than spinosad (vs. control:  $t_{20,6} = 3.86$ ;  $P = 0.001$ ; Figure 8). Without pollen, the number of adults was higher in the control compared to chlorpyrifos ( $t_{20,6} = 3.20$ ;  $P = 0.004$ ; Figure 8) and spinosad ( $t_{20,6} = 4.45$ ;  $P = 0.0002$ ; Figure 8), while no differences emerged between insecticide treatments ( $t_{20,6} = 1.25$ ;  $P = 0.225$ ; Figure 8).

A positive effect of pollen was found regarding *K. aberrans* juveniles (Table 2). The presence of juveniles was affected by insecticides (Table 2), with the same trend as for motile forms and adults. There was a significant interaction between pollen application and insecticides, together with the third order interaction “pollen\*insecticides\*time” (Table 2). After treatment a positive effect of pollen was found in control ( $t_{85,6} = 3.63$ ;  $P < 0.001$ ; Figure 8) and chlorpyrifos ( $t_{85,6} = 5.14$ ;  $P < 0.001$ ; Figure 8), but not in spinosad plots ( $t_{85,6} = 1.57$ ;  $P = 0.121$ ; Figure 8). In particular, with pollen application only a negative effect of spinosad (vs. control:  $t_{85,6} = 6.55$ ;  $P < 0.0001$ ; vs. chlorpyrifos:  $t_{85,6} = 5.50$ ;  $P < 0.0001$ ; Figure 8) was found, while no differences emerged between chlorpyrifos and control ( $t_{85,6} = 1.05$ ;  $P = 0.2987$ ; Figure 8). Without pollen application, juvenile numbers were higher in the control compared to chlorpyrifos ( $t_{85,6} = 2.25$ ;  $P = 0.012$ ; Figure 8) and spinosad ( $t_{85,6} = 4.49$ ;  $P < 0.0001$ ; Figure 8), while no differences emerged between insecticide treatments ( $t_{85,6} = 1.94$ ;  $P = 0.056$ ; Figure 8).

## Discussion

Cattail pollen resulted as being an adequate food source for predatory mites in terms of population persistence and increase. *Kampimodromus aberrans* fed with pollen exhibited a better performance compared to those fed with prey. In laboratory experiments with pesticides, the provision of a high amount of pollen and the increase of pollen application frequency augmented predatory mite fecundity. These results confirmed that predatory mite fecundity depends on food amount and stress the importance of the freshness of food provided by frequent pollen application. The effect of pollen application frequency was independent from the pollen amount provided. Potted plant experiments corroborated the positive effect of pollen on predatory mite population: pollen application induced higher predatory mite population levels. This effect was evidenced by adults and juveniles. These results confirmed the importance of pollen as food source for *K. aberrans* (Schausberger, 1992; Kreiter *et al.*, 2002; Kasap, 2005; Ozman-Sullivan, 2006; Lorenzon *et al.*, 2012). When prey availability diminishes, the most frequent food source for predatory mites is often windborne pollen and thus predatory mite and pollen abundances can be strictly related (Engel and Ohnesorge, 1994b; Addison *et al.*, 2000; Duso *et al.*, 2004). Pollen is an important source of proteins, lipids, carbohydrates and minerals (Lundgren, 2009). However, the nutritional value of pollen can degrade over time (Vanbilsen and Hoekstra, 1993; Lundgren, 2009). The results we obtained showed that predatory mites gain an advantage from feeding on fresh pollen. The beneficial effects of pollen augmentation can be observed in a wide range of natural enemies and its augmentation as a practice should be considered for conservation biological control (Gurr *et al.*, 2004; Wackers and van Riijn, 2005; Landis *et al.*, 2000; Montserrat *et al.*, 2012).

Natural enemies of importance in agriculture can be endangered by the lethal and sub-lethal effects of pesticide use (e.g., Lester *et al.*, 1999; Desneux *et al.*, 2007; Poletti *et al.*, 2007; Duso *et al.*, 2008; Beers *et al.*, 2009; Bostanian *et al.*, 2009, 2010; Bernard *et al.*, 2010). We showed the detrimental effects of spinosad and chlorpyrifos on *K. aberrans*. Both insecticides reduced the survival of this predator, but the mortality caused by chlorpyrifos was less dramatic than that by spinosad. Fecundity was also reduced by insecticides. No eggs were laid by the few females that survived spinosad, while chlorpyrifos caused a significant reduction in fecundity. The *K. aberrans* strain used in the experiment was collected in commercial orchards where organophosphate insecticides were widely applied and showed resistance to pesticides of this class (Tirello *et al.*, 2012). Spinosad is a relatively new insecticide (Thompson *et al.*, 2000) that has been used less in apple orchards than



organophosphates. Resistance to this insecticide in predatory mites has not yet been observed. Potted plant experiments confirmed the detrimental effects of insecticides, but with a lower magnitude of the effects than that seen in the laboratory. This was not surprising as laboratory experiments are often considered as a worst-case scenario where exposure is maximized (Candolfi *et al.*, 2000).

The results obtained highlighted the role of pollen in influencing the impact of pesticides on predatory mites. In laboratory trials the increase in frequency of pollen application compensated for the negative effect of chlorpyrifos on fecundity. This effect was not observed with spinosad. Apart from the effect of pollen amount, the availability of fresh pollen appears to be of particular importance. Having a higher nutritional value, fresh pollen compensated for the insecticide effects and promoted the reproduction of predatory mites. To our knowledge this is the first report on the positive effect of food sources in mitigating the effect of pesticides treatment.

The level of exposure to insecticides can also be involved in the response to fresh pollen applications. It is known that organic compounds such as pesticides can be adsorbed by pollen grains (Villa *et al.*, 2000; Thio *et al.*, 2011). This is likely to increase with the time of contact with insecticide residues. Predatory mites feeding on contaminated pollen are exposed to insecticides through ingestion. This can determine multiple exposures to pesticides with an increased negative effect on arthropods at sub-lethal level (Banken and Stark 1991; Galvan *et al.*, 2006; Pozzebon *et al.*, 2011). The provision of uncontaminated fresh pollen decreased the exposure to chlorpyrifos with a reduction in sub-lethal effects.

This effect was also clear on potted plants. At the end of the experiment, the number of predatory mites on pollen treated plants was similar between control and chlorpyrifos (Figure 8). The positive response to pollen application on chlorpyrifos treated plants emerged first in juveniles and then in adults, confirming the role of the effect on fecundity. Laboratory experiments showed that survival was not influenced by pollen provision while fecundity was. On potted plants receiving pollen, no effect of chlorpyrifos was detected on juveniles during the experimental period, whilst without pollen application this insecticide caused a population decrease to the same level as spinosad.

The effect of fresh pollen application on the fecundity of female predatory mites treated with different pesticides appears to be related to their toxicity. In the laboratory chlorpyrifos induced a reduction in fecundity, but the few females that survived spinosad application were unable to produce eggs. The reduction in fecundity of natural enemies due to insecticides use

can be related to physiological and behavioral effects such as the disruption of foraging, food processing and biochemical mechanisms involved in food conversion into egg biomass (Desneux *et al.*, 2007). The results suggest that the mechanisms that regulate the fecundity of predatory mites were completely compromised by spinosad application, and treated females were unable to gain an advantage from the availability of fresh pollen. This aspect is clear in the potted plants experiment where, after spinosad application, *K. aberrans* population persisted at low level and independently of pollen applications. On the other hand on chlorpyrifos treated plants, pollen applications induced a population growth in line with the potential of cattail pollen shown in the first laboratory experiment and in previous studies (Lorenzon *et al.*, 2012). It should be noted that most of the life-history parameters are temperature dependent (Broufas *et al.*, 2007) and the potted plant experiment was not conducted at constant temperature. The trend in juveniles increase from 3 to 7 days after treatment observed on chlorpyrifos treated with pollen was higher than in the control. This provides further support that the effect of pollen application is mediated by fecundity. Fecundity in *K. aberrans* and other predatory mites is density-dependent (Kostiainen and Hoy, 1994; Malison, 1996). The density of predatory mites was reduced by chlorpyrifos but the surviving females exhibited a fecundity level that overcompensated for the detrimental effect of the insecticide.

Food subsidy practices (e.g., food spray, habitat management) can be applied to enhance the population of natural enemies (Bostanian *et al.*, 2004; Begum *et al.*, 2006; Wade *et al.*, 2008) and are considered key aspects in innovative pest management practices (Simpson *et al.*, 2011a, 2011b). However, pesticide use poses major threats to successful biological control. Pesticides are often necessary to control key pathogens and pests in agricultural crops, and they are sometimes required for quarantine pest eradication. Their use can have dramatic effects on the environment, and can reduce the biological control potential (Tilman *et al.*, 2001; Geiger *et al.*, 2010). The reduction of pesticide side-effects on non-target arthropods is a prerequisite for any conservation practice in agro-ecosystems; but the findings obtained here stress that management practices aimed at season-long food availability can mitigate the effect of pesticides on natural enemies, promoting their persistence in agro-ecosystems.

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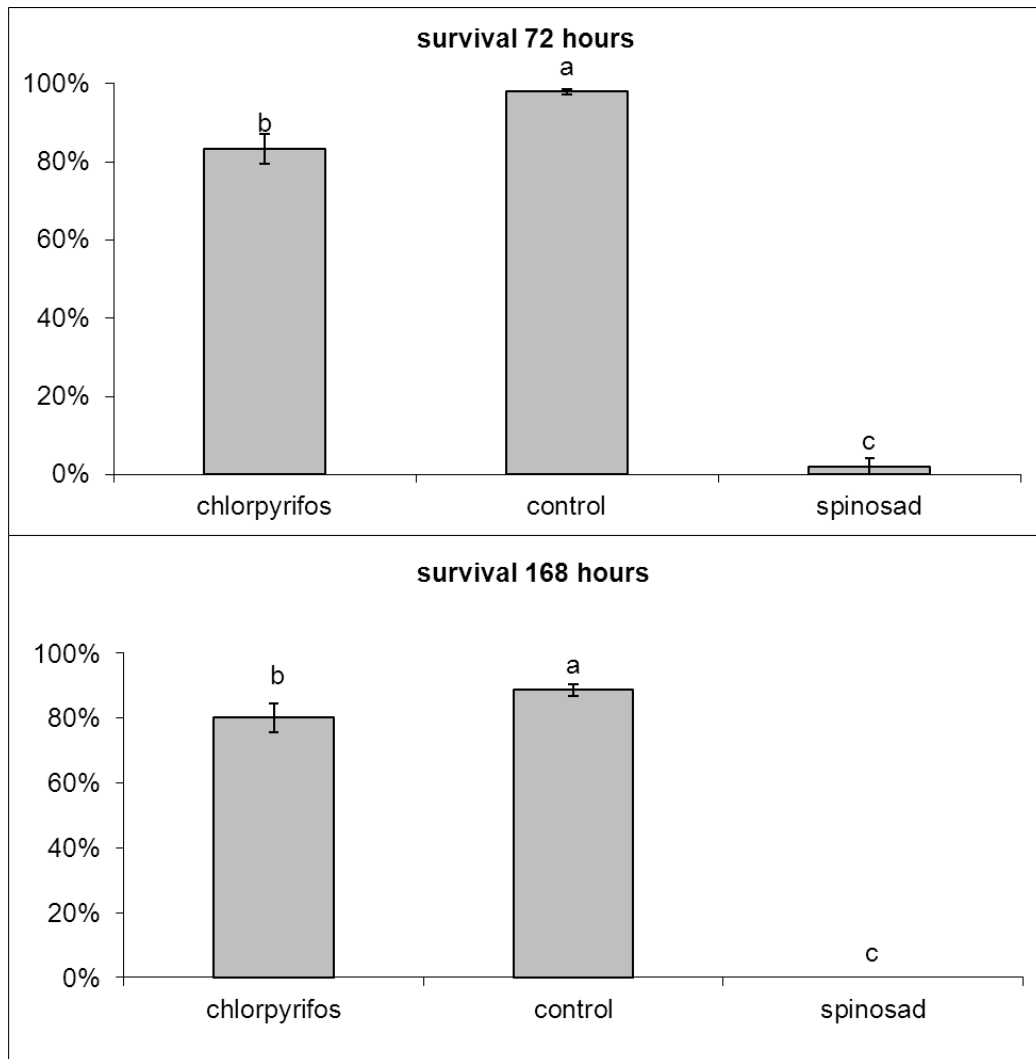


Figure 1- Survival of *K. aberrans* females treated with insecticides measured after 72 and 168 hours. Different letters indicate significant differences at Wald chi-square test ( $\alpha = 0.05$ ).

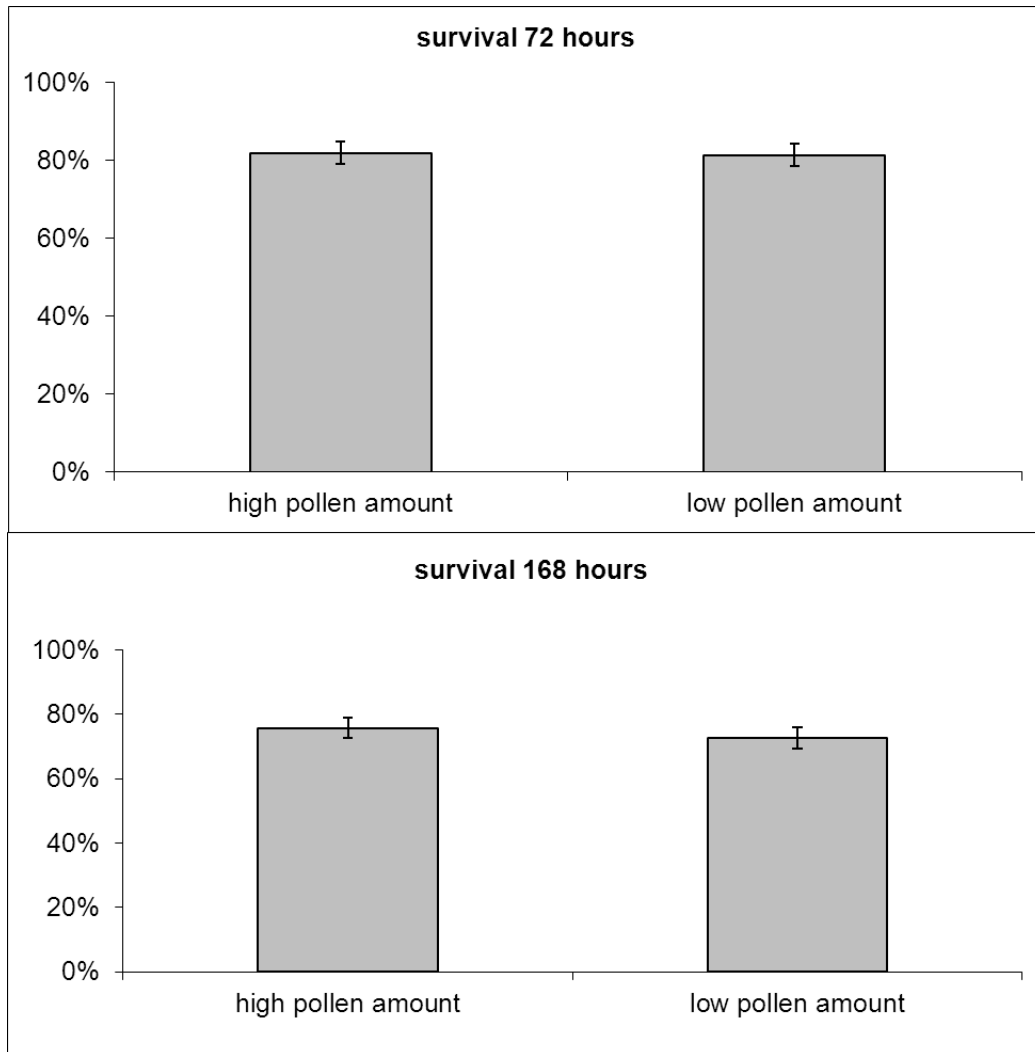


Figure 2 - Survival of *K. aberrans* females receiving different amount of pollen measured after 72 and 168 hours. Different letters indicate significant differences at Wald chi-square test ( $\alpha = 0.05$ ).

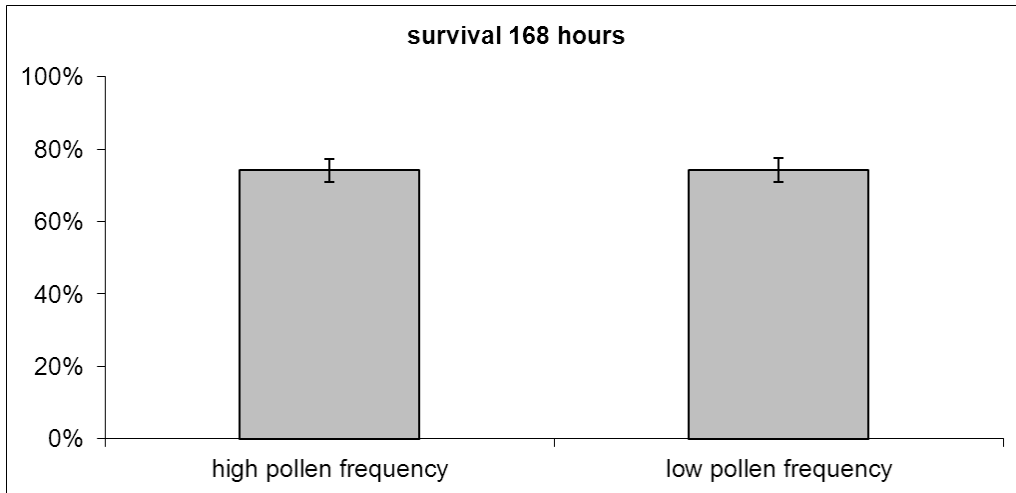


Figure 3 - Survival of *K. aberrans* females receiving different pollen application frequency measured after 168 hours. Different letters indicate significant differences at Wald chi-square test ( $\alpha = 0.05$ ).

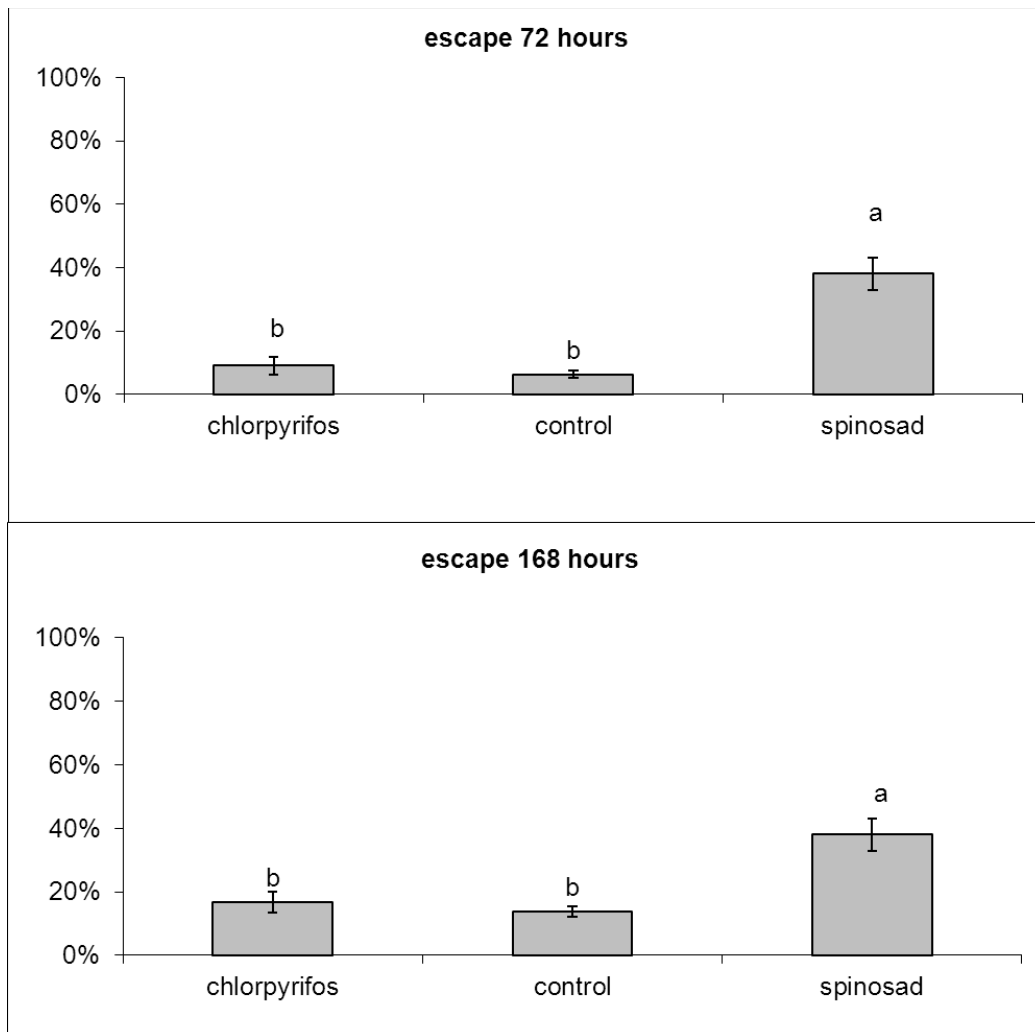


Figure 4 - Escape rate of *K. aberrans* females treated with insecticides measured after 72 and 168 hours. Different letters indicate significant differences at Wald chi-square test ( $\alpha = 0.05$ ).

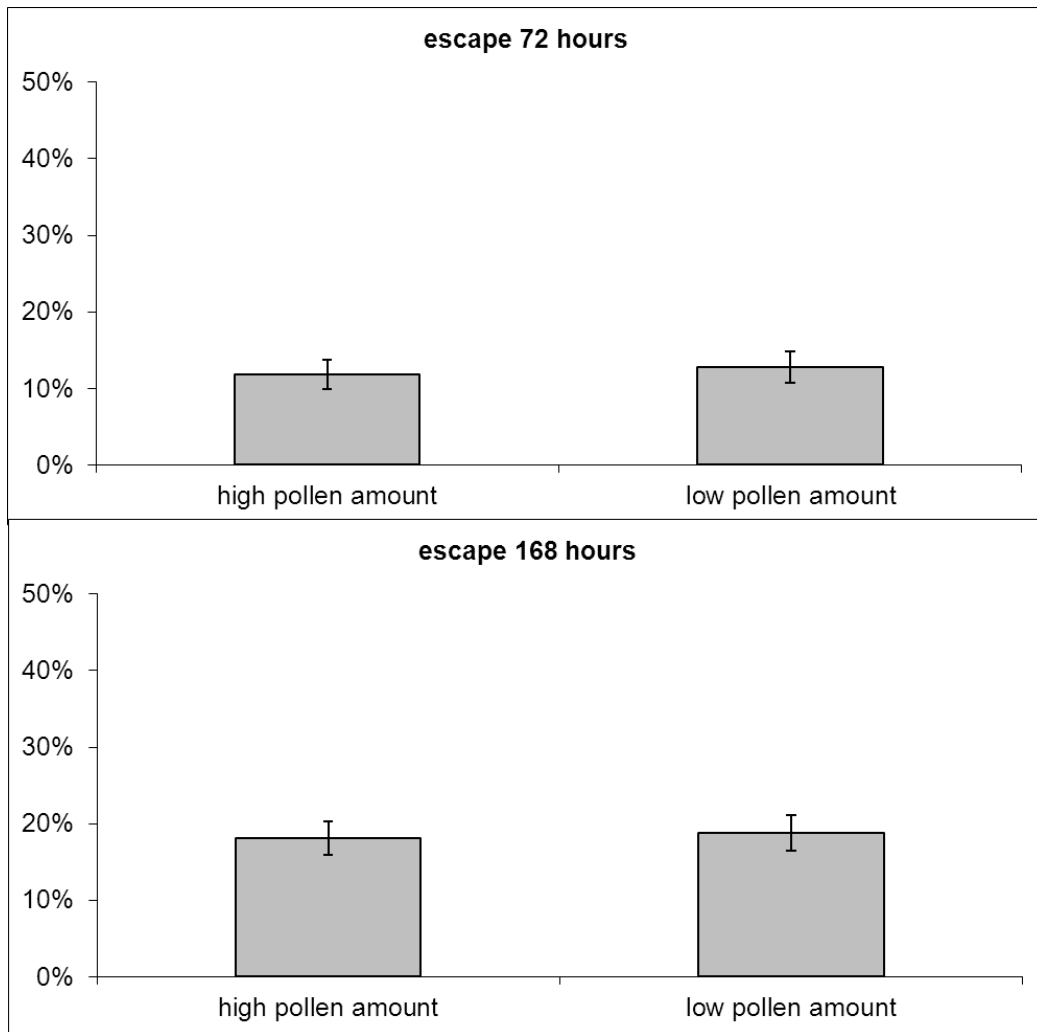


Figure 5 - Escape rate of *K. aberrans* females receiving different amount of pollen measured after 72 and 168 hours. Different letters indicate significant differences at Wald chi-square test ( $\alpha = 0.05$ ).



Figure 6 - Escape rate of *K. aberrans* females receiving different pollen application frequency measured after 168 hours. Different letters indicate significant differences at Wald chi-square test ( $\alpha = 0.05$ ).



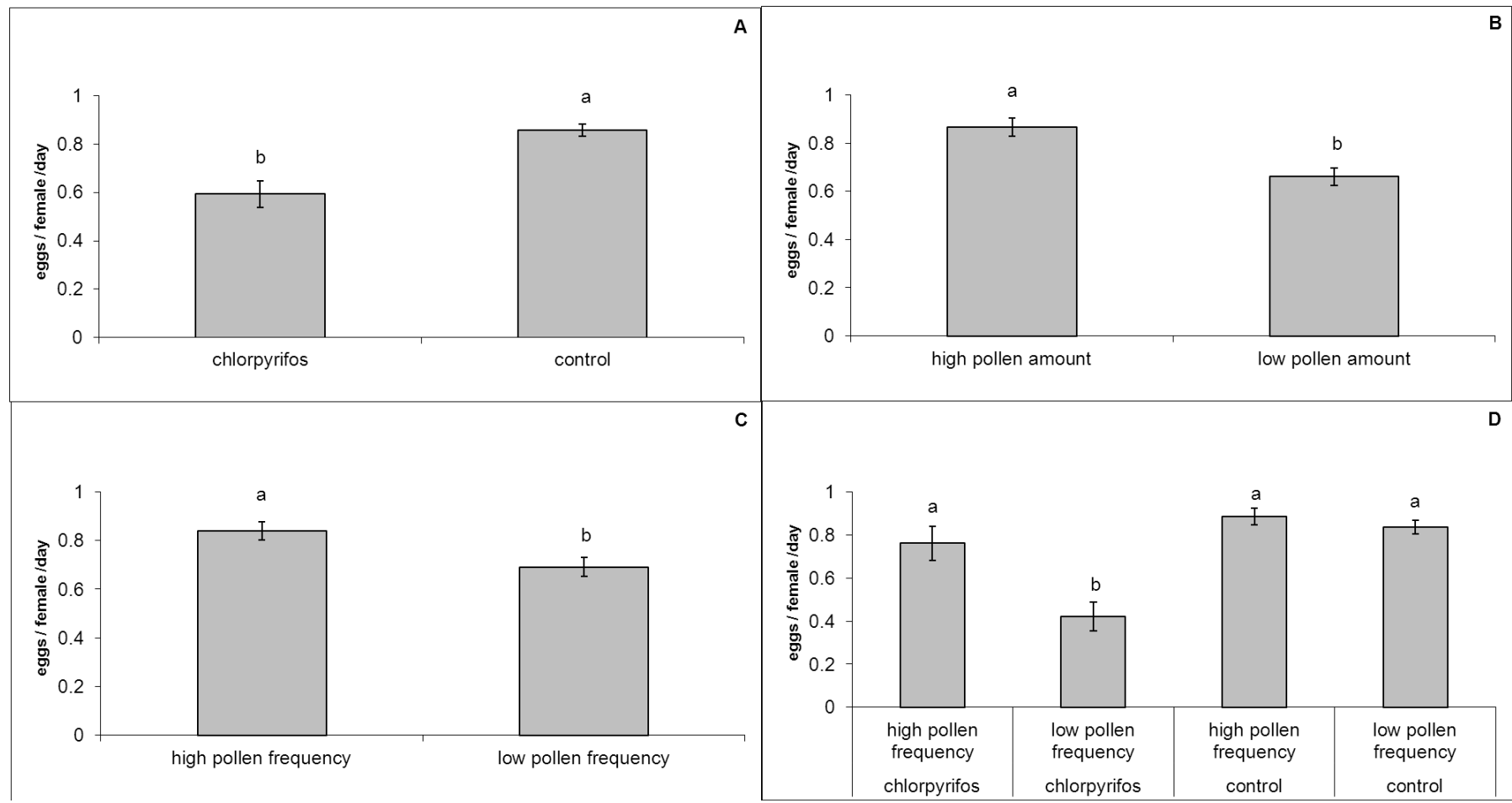


Figure 7 - Fecundity in 168 hours of *K. aberrans* females treated with insecticides (A), receiving pollen amount (B), different pollen application frequency (C) and treated with insecticides and different pollen application frequency (C). Different letters indicate significant differences at t-test ( $\alpha = 0.05$ ).

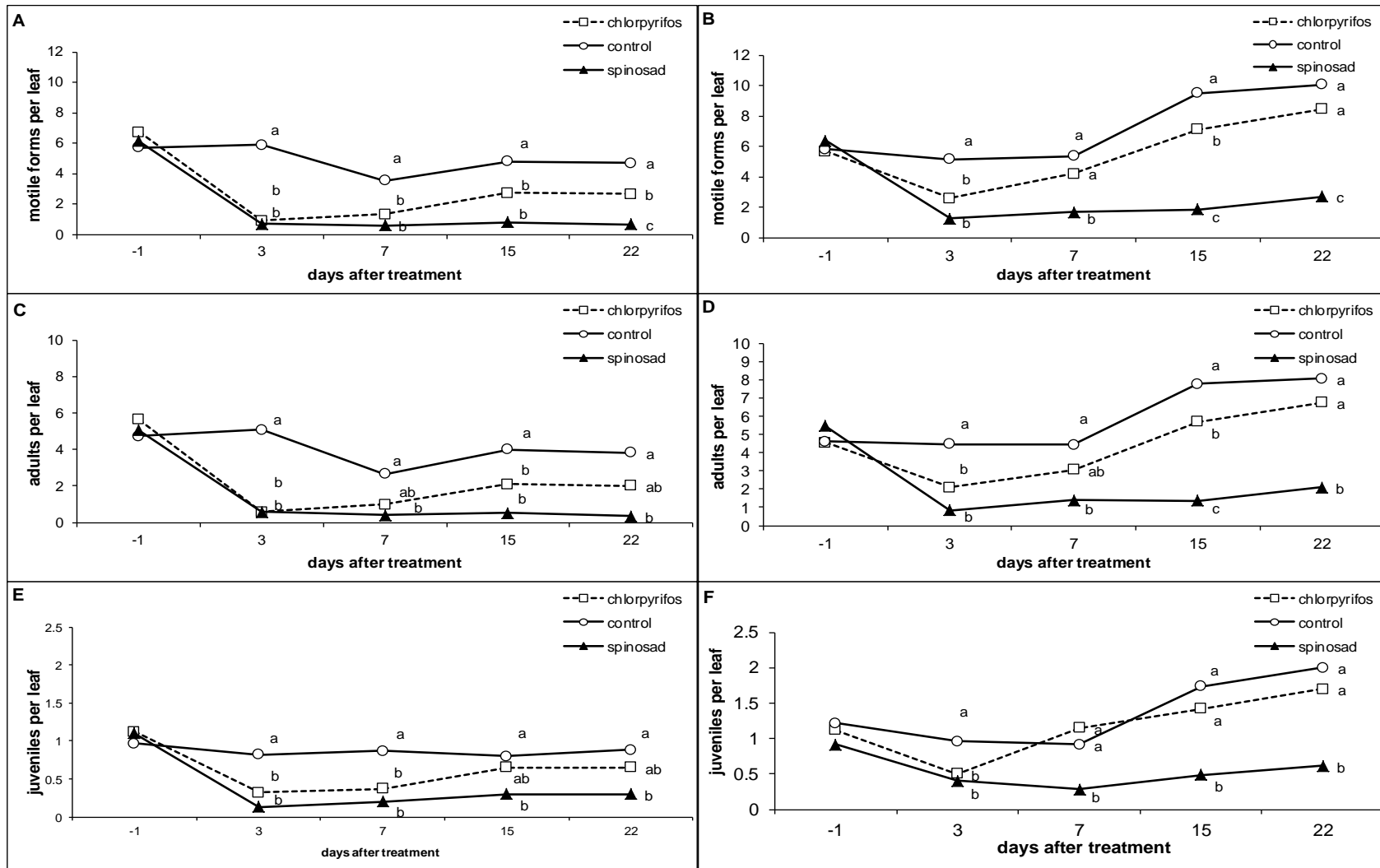


Figure 8 - Density of *K. aberrans* motile forms (A, B), adults (C, D) and immatures (E, F) observed on plant receiving insecticides and pollen application. Figure A, C and E represent data from no pollen treatments, while B, D and F are obtained from pollen treatments data. Different letters indicate significant differences at t-test ( $\alpha = 0.05$ ) to the least-square means.

**Table 1** - Life-table parameters ( $\pm$ SE) of *K. aberrans* reared on cattail pollen and *Panonychus ulmi*.

Parameters	Cattail pollen	<i>Panonychus ulmi</i>
Dt (day)	6.180 $\pm$ 0.222 b	14.040 $\pm$ 0.959 a
$\lambda$ (female/female/day)	1.119 $\pm$ 0.005 a	1.050 $\pm$ 0.113 b
$r_m$ (female/female/day)	0.112 $\pm$ 0.004 a	0.049 $\pm$ 0.003 b
$R_o$ (female/female)	10.943 $\pm$ 0.583 a	2.696 $\pm$ 0.277 b
T (day)	21.355 $\pm$ 0.601	20.327 $\pm$ 1.235

Values within a row bearing different letters were significantly different a t-test ( $\alpha = 0.05$ ).

**Table 2** - Results of REML repeated measures analysis with *Kampimodromus aberrans* population density as dependent variable. Degrees of freedom in all models were calculated using the Kenward-Roger method.

Source of variation	F	d.f.	p
<i>Motile forms</i>			
Pollen	17.94	1, 18.4	0.001
Insecticides	21.52	2, 18.4	<.0001
Pollen*Insecticides	1.22	2, 18.4	0.319
Time	73.81	4, 243.0	<.0001
Pollen*Time	17.63	4, 243.0	<.0001
Insecticides*Time	21.36	8, 253.0	<.0001
Pollen*Insecticides*Time	3.79	8, 253.0	0.000
<i>Adults</i>			
Pollen	13.91	1, 18.5	0.002
Insecticides	17.81	2, 18.5	<.0001
Pollen*Insecticides	0.82	2, 18.5	0.455
Time	62.81	4, 244.0	<.0001
Pollen*Time	14.12	4, 244.0	<.0001
Insecticides*Time	21.37	8, 254.0	<.0001
Pollen*Insecticides*Time	3.47	8, 254.0	0.001
<i>Juveniles</i>			
Pollen	28	1, 75.8	<.0001
Insecticides	25.94	2, 75.8	<.0001
Pollen*Insecticides	3.14	2, 75.8	0.049
Time	21.56	4, 237.0	<.0001
Pollen*Time	6.36	4, 237.0	<.0001
Insecticides*Time	3.06	8, 250.0	0.003
Pollen*Insecticides*Time	2.32	8, 250.0	0.021

## Chapter VI

### **Acetylcholinesterase cDNA cloning in chlorpyrifos susceptible and resistant strains of the predatory mite *Kampimodromus aberrans* (Acari: Phytoseiidae).**

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The manuscript in preparation as:

Stefano Cassanelli, Shakeel Ahmad, Carlo Duso, Alberto Pozzebon, Paola Tirello - Target site resistance to chlorpyrifos in insensitive Acetylcholinesterase (AChE) in *Kampimodromus aberrans* (Acari: Phytoseiidae) from North-eastern Italy

I contributed to the laboratory experiments and data analysis

## Introduction

### Acetylcholinesterase genes in insects and mites

Acetylcholinesterase (AChE, EC 3.1.1.7) is an enzyme anchored on the surface of post synaptic membranes at cholinergic synapses and neuromuscular junctions. AChE terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine in the cholinergic nervous system of most animals, including insects and mites (Figure 1). Beyond neuronal transmission, AChE is thought to play other roles, such as neurite growth and synapse formation, modulation of glia activation and learning/memory (Shapira *et al.*, 2001; Soreq and Seidman 2001).

An extensive phylogenetic and biochemical analyses on cloned AChE gene/s suggest that before the differentiation of insects, a duplication event of an ancestral gene led to the development of two AChE loci encoding for AChE1, which is paralogous to *Drosophila* AChE, and AChE2 the orthologous to *Drosophila* AChE (Kim and Lee, 2012). By examining 100 insect species, more than half possess both AChEs, with AChE1 responsible for the main catalytic activity. However in some species ranging from Palaeoptera to Hymenoptera, AChE1 locus is lost and only AChE2 was detectable at the genomic and functional level (Kim and Lee, 2012; Huchard *et al.*, 2006).

In Acari subclass the number of AChE loci has not been investigated so deeply. The recent analysis of spider mite *Tetranychus urticae* (Acari: Tetranychidae) genome revealed only one locus encoding for a functional AChE (Grbic *et al.*, 2011), excluding the contributing of other paralogous AChE(s) genes to AChE activity. This finding was supported by a unique AChE transcript detected after transcriptomic analysis of the citrus red mite, *Panonychus citri* (Niu *et al.*, 2012). However in the cattle tick *Rhipicephalus (Boophilus) microplus*, three different cDNAs encoding for putative AChEs, were functionally characterized, by using an *in vitro* baculovirus expression system (Temeyer *et al.*, 2010). Likewise transcriptome analysis in American dog tick, *Dermacentor variabilis* (Acari: Tetranychidae) revealed seven AChE transcripts, probably expressed from different loci, relating on their diverging nucleotide sequences (Bissinger *et al.*, 2011). The evidence that tick genomes, contain multiple copy of AChE genes came from the annotation of the black-legged tick *Ixodes scapularis* genome, where several AChEs loci were predicted, even not yet functionally confirmed (Van Zee *et al.*, 2007).

To date, no studies on AChE were reported for the Phytoseiidae family. Recently the genome project of the Western predatory mite *Metaseiulus occidentalis* has been completed (Western Orchard Predatory Mite Genome Project, 2012) and the automatic annotation phase started. Preliminary *in-silico* gene prediction by GNOMON algorithm, using previously cloned or annotated mRNA sequences for arthropod AChEs, supports the evidence for multi-loci AChEs in *M. occidentalis*.

### **AChE target site resistance in insects and acari**

Pesticide resistance in insects and mites is a serious worldwide problem in agriculture (Pesticide Resistance Database, 2012). Resistance is often caused by the overuse and misuse of pesticides leading to metabolic and/or target site resistance.

Target site resistance rises from gene mutations at the target for the insecticide, resulting in insensitivity to the active principle. It is usually monogenic but it can be polygenic too if multiple mutated copies of the same gene exist or when different mutated subunits are assembled into the same insecticide target molecule (e.g. nicotinic acetylcholine receptors or ion channel units).

Organophosphates (OPs) and Carbamates are two important classes of inhibitors which act as analogous to the substrate acetylcholine, inactivate the AChE by phosphorylating or carbamylating a serine residue in the enzyme's catalytic centre (Figure 2); Ishaaya *et al.*, 2001). The neuro toxic effect of AChE inactivation is then caused by repetitive firings of postsynaptic leading to a lethal desensitization of the nervous system.

Many different mutations, six in AChE1 and eleven in AChE2, have been associated with insensitivity to OPs and carbamates mainly in insect and a few mite species (Russel *et al.*, 2004; Khajehali *et al.*, 2010; Temeyer *et al.*, 2009). Nevertheless, the range of insensitivity to different compounds, as magnitude and spectra, is highly variable among species and even inside the same specie. The differences in resistant phenotype can be due to the presence of more than one gene encoding for multiple AChE targets and by the presence of overlapping metabolic resistance. Through three dimensional modelling most of mutations are thought to reduce the AChE sensitivity by providing steric hindrance to the insecticide entrance at the catalytic site, while allowing the access of the smaller acetylcholine substrate. Two examples of these recurrent mutations are G119S and F331W (AChE amino acid numbering from *Torpedo californica* mature AChE) (Russell *et al.*, 2004; Oakeshott *et al.*, 2005). These mutations were found, alone or in association, affecting the AChE gene in *T. urticae* strains resistant to several OPs and one carbamate (Khajehali *et al.*, 2012). Both mutations are

responsible for a decreasing in AChE sensitivity to the OPs monocrotophos and chlorpyrifos at the price of a reduction in enzyme's catalytic efficiency (Kwon *et al.*, 2012; Khajehali *et al.*, 2012) with an additive effect when the G119S and F331W substitutions were present simultaneously in *in vitro* expressed *T. urticae* AChE (Kwon *et al.*, 2012). These findings suggest that the co-selection of both mutations can be favoured in field condition since they act in a synergistic manner boosting resistance phenotype. In addition some amino acid substitutions outside the catalytic site may have essentially a further additive effect, increasing the stability of the enzyme and its ability to hydrolyse the acetylcholine (Mutero *et al.*, 1994; Shi *et al.*, 2004), counteracting the inhibitory effects of the insecticides and/or the reduction in catalytic efficiency due to co-existing AChE mutations. This is the case of A391T mutation in *T. urticae* AChE, which was found, associated to the F439W substitution (analogous to F331W) in field populations resistant to monocrotophos (Kwon *et al.*, 2010b, 2012). Another adaptive response in *T. urticae* may consist in AChE gene duplication (Kwon *et al.*, 2010a, 2012) since multi-copies of a gene per aploid genomes increases the chance that mutations conferring insecticide resistance can be positive selected. Indeed the co-presence of wild type and mutated gene copies and/or an increased transcription rate may also compensate the fitness cost due to a reduced catalytic efficiency of insensitive AChEs. The same evolution strategy is probably adopted by resistant strain of thick *Rhipicephalus Microplus*, which displays elevated copy numbers of the three AChE loci (Temeyer *et al.*, 2009, 2012).

The metabolic resistance can also contribute to the resistant phenotype. It consists in an efficiency increase of an elaborate three-phase detoxification system, metabolizing xenobiotics into less harmful substances and facilitating their excretion (Xu *et al.*, 2005). It is usually under polygenic control since involves multi-gene family enzymes encoding for

- a) P450 monooxygenases, which decrease the biological activity of a broad range of substrates (or, increase their toxicity as well, i.e. chlorpyrifos activated in oxon form), phase I.
- b) Glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), which adding bulky side groups onto toxic compounds increase their hydrophilicity, facilitating the excretion from the organism (phase II). Carboxylesterases (EST) which catalyze the hydrolysis of ester-containing xenobiotics (such as organophosphate insecticides), leading to their detoxification, phase II.

ATP-binding cassette (ABC) and other transmembrane transporters that actively export the conjugated toxins out of the cell into an excretory system, phase III.

Likely metabolic resistance accounts for difference sensitivity to chlorpyrifos and dimethoate in *T. urticae* populations sharing the same F331W substitution in their AChE (Khajehali *et al.*, 2010). Main objective of our study was cloning in the predatory mite *Kampimodromus aberrans* the homologous AChE cDNA characterized in *T. urticae* and looking for new and/or previously described mutations affecting the AChE sensitivity to chlorpyrifos, in resistant strain of *K. aberrans*.

## **Materials and Methods**

### ***Kampimodromus aberrans* populations**

The study was performed on four resistant and three susceptible *K. aberrans* strains collected in North-eastern Italy. Resistant strains include; 1) Posenato that was collected from a commercial vineyard located at Monteforte d'Alpone (Verona province, Veneto region), where predatory mites showed a low susceptibility to OPs (Posenato 1994); population Meneghello (2) was collected from a commercial vineyard located in Treviso province (San Vito di Valdobiadene, Posas). Resistant populations (3) San Floriano and Bixio (4) were collected from a commercial vineyard (Verona province, Veneto region). From 1997 to 2008, OP (chlorpyrifos) was used every year at Monteforte d'Alpone but much less frequently (1-2 times in the last 1990s) at San Vito di Valdobiadene, Posas. Three susceptible populations including; 1) population Paese (located at Treviso province), (2) population Padova and (3) population Legnaro were collected from untreated hackberry trees (*Celtis australis* L.) at Legnaro (Padova province, Veneto region). We assumed Paese population as reference for its susceptibility to organophosphate compounds. These populations were collected and reared for several generations in separate boxes in the laboratory at the Department of Environmental Agronomy and Crop Science, University of Padova. Grapevine leaves were used as substrate for predatory mites. They were settled on a pad of wet cotton where small pieces of PVC were placed for shelter and oviposition. *Typha latifolia* pollen was provided every two days as food.

### **Insecticide bioassays**

Bioassays were conducted with a chlorpyrifos formulation, (Dursban® 75 WG, 75% a.i., Dow AgroSciences, 70 g/hl) widely used in vineyards to control leafhoppers (e.g., *Scaphoideus titanus* Ball. *Empoasca vitis* Göthe), grape berry moths (*Lobesia botrana* Den. & Schiff. and *Eupoecilia ambiguella* Hübner) and mealybugs (e.g. *Planococcus ficus*



Signoret). This formulation was diluted into distilled water before testing procedures. Toxicological trials were performed by using rectangular leaf sections (6 cm<sup>2</sup>). They were immersed in the insecticide solution for 30 sec. and then left to dry. Control leaf units were immersed into distilled water for 30 sec. Leaf sections were put on a wet cotton pad and cotton barriers were created along their perimeter to avoid predatory mite escape. Two mated *K. aberrans* females (about 12 days old) were gently transferred on each leaf section using a fine brush and fresh pollen was provided every two days as food. Units were kept into a climatic chamber at 25 ± 2° C, 70 ± 10% relative humidity and 16L: 8D photoperiod. Female mortality was assessed 72 h after treatments. Females drowned or escaped were removed from the initial tested number.

Before proceeding towards Total RNA extraction, 200 adult females of each *K. aberrans* tested population were collected in Appendorf vials and stored at -80°C.

### **Primer design for cloning AChE cDNA in *K. aberrans***

The annotated version of the genome assembly (release Mocc\_1.0, March 2012) of western orchard predatory mite *M. occidentalis* was used to look for *putative* for AChE-like proteins, through tBlastn algorithm (<http://www.ncbi.nlm.nih.com>), using the cDNA AChE cloned in *T. urticae* (GenBank accession n. ADK12685.1) as query sequence.

Predicted mRNAs coding for putative AChE-like were extracted from scaffolds and their open reading frames (ORFs) compared to *T. urticae* AChE protein using Lasergene sequence analysis tools EditSeq and MegAlign 5.0 (DNASTAR, Inc., Madison, WI, USA).

Degenerate primers were designed by manual inspection of conserved domains after alignments of *T. urticae* AChE and putative orthologous AChE-like proteins in *M. occidentalis*. The resulting primers were used to amplify cDNA core fragments of homologous AChE in *K. aberrans*. No degenerate primers necessary to complete the cDNA cloning, by walking steps and 3'-5' RACEs in *K. aberrans* were outlined with PrimerSelect 5.0 (DNASTAR, Inc., Madison, WI, USA).

### **mRNA extraction and AChE cDNA cloning**

Total RNA was extracted homogenising 200 adults in 500 µl Tri-Reagent (Sigma), according to the manufacturer's instructions. Sample's integrity was checked in electrophoresis on a 1.2% agarose, 2.2 M formamide/formaldehyde denaturing gel. Quality and quantity assessment of the extracted RNA were performed in Nanodrop ND-1000 Spectrophotometer (NanoDrop, Fisher Ther-mo, Wilmington, DE, USA). First strand cDNA

was synthesized according to the supplier recommended protocol, using Improm-II reverse transcriptase (Promega) and random primers. Amplification of a cDNA fragment for a putative AChE in *K. aberrans* was achieved through two consecutive rounds of RT-PCR, employing degenerate primers. PCR mixtures (25  $\mu$ l) contained GoTaqGoTaq Flexi buffer 1x buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 30 pmol forward and reverse degenerate primers, 1 U GoTaq, 2.5  $\mu$ l of cDNA. Degenerate primers laid on conserved functional domains of homologous AChEs proteins in *T. urticae* and *M. occidentalis*. For the first RT-PCR round the forward and reverse primers are KaAChEF1 (GIPYAKP domain) and KaAChER1 or KaAChER2 (WVYGGSF motif) respectively (Table 1). This PCR was then 10 fold diluted and used as template for a second RT-PCR where the KaAChEF1 primer was replaced with a more internal primer, KaAChEF2, designed on PYAKPP domain (Table 1). The two PCR rounds share same thermal profile, consisting in an initial denaturation step of 3 min at 94 °C followed by 5 cycles at 94 °C for 30 s, 45 °C for 30 s, 72°C for 60 s, then 5 cycles at 94 °C for 30 s, 45 °C plus +1 °C/cycle, 72°C for 60 s, and further 25 cycles at 94 °C for 30 s, 45 °C for 30 s, 72°C for 60 s, with a final extension step at 72 °C for 10 min. PCR products of expected size (around 300 bp) were purified from 1% (w/v) agarose/TBE 1x gel using EuroGOLD Gel Extraction Kit (Euroclone) and cloned using pGEM-T easy vector (Promega). Plasmids were purified with EuroGOLD Plasmid Miniprep Kit (Euroclone) and sent for sequencing at the BMR genomics (Padua, Italy). Sequences were assembled and analysed using SeqMan 5.0 (DNASTAR, Inc., Madison, WI, USA). Identification of AChE-like sequences was performed with BLASTX search in GenBank (<http://www.ncbi.nlm.nih.gov>) using the ORFs deduced from the cloned cDNA fragments. The cDNA cloning was further extended in 3' direction by performing a RT-PCR which used a forward primer designed on the first cloned cDNA fragment in *K. aberrans* (KaAChEF3) and a reverse primer (KaAChER3) devised on the conserved AChE domain CAFWKNFL in *M. occidentalis* (Table 1). The PCR mixture has the same composition described above except primer concentration was reduced to 15 pmol. PCR was carried out in 1 cycle of 94 °C for 2 min, followed by 5 cycles including three steps of 94°C for 30 s, 50 °C for 30 s, 72 °C for 60 s, 5 cycles of 94 °C for 30 s, 50 °C for 30 s (+ 1 °C/cycle) , 72 °C for 60 s, 20 cycles of 94°C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension step at 72 °C for 10 min. PCR product was purified, sequenced and analyzed as described above. 3' and 5' Rapid Amplification of cDNA ends reactions were performed to complete the cDNA sequences of *AChE* (Figure. 3). In RACE reactions the first strand cDNA was synthesized using total RNA and polyT-adaptor primer for 3' RACE or KaAChE-R4 for

5' RACE (Table 1), according to the manufacturer protocol (5' RACE System for Rapid Amplification of cDNA Ends, Invitrogen). The 3' RACE product spanning across the unknown 3'-end of *AChE* was amplified in two consecutive PCR rounds, coupling the primers KaAChEF4 and Adaptor1 and KaAChEF5 and Adaptor2. For obtaining the 5' end of AChE transcript, the cDNA went through polyC-tailing of its 3'-end by terminal deoxynucleotidyl-transferase (TdT) following the kit protocol (5' RACE System for Rapid Amplification of cDNA Ends, Invitrogen). The upstream cDNA sequence encompassing the 5' untranslated region were obtained with two PCR rounds using the couples of primers KaAChER5-TS-primer and KaAChER6-TS-PCR (Table 1). The first 5'RACE round was done at 94 °C for 2 min (1 cycle), 5 cycles at 94 °C, 56 °C for 30 s, 72 °C for 60 s, 5 cycle at 94 °C, 57 °C for 30 s, 72 °C for 60 s, and 20 cycles at 94 °C for 30 s, 58 °C for 20 s, 72 °C for 60 s. The second 5'RACE round consisted in 1 cycle at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s. The 5' RACE fragment was purified from agarose gel and sequenced as previously described.

### **Full length AChE cDNA sequencing**

Total RNA was extracted from adults of both susceptible (Paese) and resistant (Posenato) strains with Tri-Reagent as done for cDNA cloning. First strand cDNA was synthesized from total RNA with Improm reverse transcriptase (Promega) with random primers as indicated by manufacturer's protocol. To sequence the ORF of the cloned cDNA, three RT-PCR fragments partially overlapped were amplified using the following couple of primers KaAChEF6-R7, KaAChEF7-R8 and KaAChEF8-R9 (Table 1). PCR reaction (25 µl) included 2 µl of cDNA, and a final concentration of GoTaq Flexi 1x buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.6 µM of each primers, 0.625 U/µl GoTaq (Promega). The adopted thermal profile was 94°C for 2 min (1 cycle), followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C 60 s, with a final extension step at 72 °C for 10 min. PCR products were checked by electrophoresis on 1% agarose in TBE 0.5x, purified with the EuroGOLD Cycle-Pure Kit (Euroclone) and sent to BMR genomics (Padova, Italy) for sequencing. To this aim the same primers for RT-PCR amplifications plus new internal primers (KaAChEF9, R10, and F10) were used (Table 1). Chromatograms were assembled with SeqMan tools (DNASTar, Lasergene), and the alignment of cDNA consensus sequences from sensible and resistant strains were manual inspected for non synonymous SNPs with MegAlign program (DNASTar, Lasergene). The same program provided the phylogenetic tree after clustal method alignment of AChE sequences.

## DNA extraction and screening for G119S and F331W mutations

DNA extraction was performed according to Tixier *et al.*, 2008, scaling up the reagents. Two thousands of frozen adults for each strain were homogenized in 150 µl of extraction buffer (2% CTAB, 1.4M NaCl, 0.2% 26-mercaptoethanol, 100mM EDTA, 100mM Tris-HCl, and pH 8.0) using a micro tissue grinder (Wheaton, Millville, NJ). The homogenate was transferred in a 0.5 ml test tube and incubated for one hour at 65°C, with periodical hand mixing. One hundred and fifty microliters of chloroform: isoamyl alcohol mixture (24:1) was added, mixed by inversion and tubes were centrifuged at 6°C for 5 min at 1000 g. The aqueous solution was collected in a new test tube and 80 µl of isopropanol was added to the decanted aqueous phase and chilled at -20°C for 20 min for DNA precipitation. After centrifugation (15 min, 6°C, 1000 g), the pellet was suspended in 100 µl of 96% alcohol at 4°C. After a final centrifugation of 10 min (6°C, 1000 g), the dried pellet was suspended in 30 µl of de-ionized water. Quality and quantity of extracted DNA was assayed by spectrophotometric analysis at Nanodrop ND-1000, as its integrity through electrophoresis on 1% agarose/TBE 0.5x gel. The screening for G119S and F331W substitutions was done by amplification and direct sequencing of AChE gene traits potentially bearing the mutations. Since the exon-intron organization in *K. aberrans* was unknown, a successful intron predictions was done by aligning the AChE cDNA sequence cloned in *K. aberrans* with the homologous gene sequence annotated in *M. occidentalis* (Spidey tool, <http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/spideydoc.html>). Relying on this information an exon free fragment was amplified for G199S screening, using primers KaAChEF11 and KaAChER11, while a short intron was included in the PCR fragment obtained with primers KaAChEF12 and KaAChER12, which was necessary to verify the presence of F331W substitution (Table 1). PCR mixture consisted in GoTaq Flexy buffer 1x, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.6 µM forward and reverse primers, 2 ng/µl DNA, 0.05 U/µl GoTaq (Promega). Amplification was carried out through 94 °C for 2 min (1 cycles), 5 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, 5 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 60 s, and 20 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 °, followed by a final extension step at 72 °C for 10 min. PCR products were purified and sequenced as described for cDNA sequencing using the same primers employed for the DNA amplifications.

## Results

### AChE gene in *M. occidentalis* genome

The tBlastn search on annotated genome of the predator mite *M. occidentalis* using the *T. urticae* AChE wild type sequence as query, gave back two scaffolds (GenBank accession numbers AFFJ01003151.1 and AFFJ01002402.1) containing predicted mRNAs (XR\_145413, XR\_145279) coding for putative AChEs with functional domains having high similarity indexes (e-value max 1e-078 and 1e-69) to query's ones. The two mRNA sequences differed at the predicted splicing sites and because XR\_145279 was shorter than XR\_145413 owing to an exon skipping at the 5' end of the ORF. When the *in-silico* predicted mRNAs went through Blastn interrogation of *M. occidentalis* transcriptome shotgun assembly, two cDNA fragments were retrieved (JL046593.1 and JL050556.1), confirming that they were actually transcribed. Altogether these findings suggested the both mRNAs coded for AChE homologous to that cloned in *T. urticae*, and that they could be informative for AChE cloning in *K. aberrans* as well.

### AChE cDNA in *K. aberrans*

cDNA of 2329 was isolated from sensible strain of *K. aberrans* (KaAChE). The deduced precursor was composed of 655 amino acids (Figure. 4) with a signal peptide predicted encompassing the first 32 amino acids from the amino terminal (Shen *et al.* 2007). The cloned KaAChE displayed most of the amino acids responsible for the functional integrity of the enzyme and usually well conserved both in insect and mite AChEs, i.e. KaAChE residues involved in intramolecular disulphide bonds (C139, C166, C325, C336, C471, C593), catalytic triad (S271, E395, H509), anionic sub-site (W156), oxyanion-hole (G190, G191, A274), and the acyl pocket (W304, F358 or F360, F399). The phylogenetic tree (Figure. 5) indicated that KaAChE sorted with a cluster formed by the putative AChE-like sequences annotated in genomes of *M. occidentalis* (coded by XR\_145413) and in *Ixodes scapularis* (XP\_002413212) or cloned in *T. urticae* (ADK12685). Since AChEs are divided in two groups, orthologous and paralogous to *D. melanogaster* AChE, and the *T. urticae* AChE belongs to the latter, also KaAChE could be considered in the same group. The amino acid identity with other AChEs in acari, ranged from 62% (*I. scapularis* putative AChE, XP\_002413212) to 33% (*R. microplus*, AChE3, AAP92139) and it was compatible with that observed comparing AChEs from different mite species or even AChEs from multiple loci in *I. scapularis* or *R. microplus*. The highest identity (> 93%) was for AChEs annotated in *M.*

*occidentalis*, in particular with XR\_145413, where the amino acid sequences were much more consistent at the putative splicing site than in XR\_145279. This suggests that the correct automated annotation of the AChE transcript in that locus in *M. occidentalis* genome was XR\_145413. As expected the highest divergence in amino acid sequence between KaAChE and XR\_145413 was restricted to the amino or carboxy –terminal of the protein, outside the functional domains.

### **Comparing AChE sequences among different strains**

Full length sequencing of cDNA KaAChE in susceptible (Paese) and resistant (Posenato) strains evinced a non synonymous G to A mutation at the position 687 which led to a G191S substitution into protein sequence (G199S AChE *Torpedo* numbering; Figure 5, 6). This residue took part to the oxyanion-hole, one of the functional domains for AChE activity. Susceptible and resistant strains differed also for another single nucleotide polymorphism (SNPs) at 1499 position of the cloned cDNA, a C to T transition which did not affect the codon meaning (D461; data not shown). The resistant strain was homozygous at this site, bearing only the T allele while the susceptible strain displayed both SNPs with a prevalence of C nucleotide over T alternative. The phenylalanine residue (F339) replaced by a tryptophan in chlorpyrifos resistant strains of *T. urticae* (F439W mutation, or F331W AChE *torpedo* numbering) was still conserved both in susceptible and resistant strains of *K. aberrans*. The same was for the glycine residue (G336) which was found replaced with Alanine (G328A) in F331W bearing strains of *T. urticae*. The presence of these three mutations was also checked in further two susceptible and three resistant strains of *K. aberrans*. Owing to a reduced number of available specimens the screening was performed at genomic level by amplifying fragments of AChE gene potentially carrying these mutations. In the case of F331W and G328A screening, the amplification of a short predicted intron was achieved. This finding confirmed the conservation of this splicing site between *K. aberrans* and *M. occidentalis* (Figure 5). The G191S substitution was found only in resistant strains as they lacked of both F331W and G328A substitutions.

## Discussion

In Acari, target site resistance conferring insensitivity to OPs, including chlorpyrifos, due to an insensitive AChE, has been described in of *T. urticae* and *T. kanzawai* (Aiki *et al.*, 2004; Khajehali *et al.*, 2010). A moderate chlorpyrifos resistance was associated to a G119S substitution (AChE torpedo numbering) in the single copy AChE gene of *T. urticae* while an higher level of insensitivity (> 400 folds when compared to a sensible strain) was usually detected in case of F331W substitution. Both substitutions conferred high OP and carbamate resistance in mosquito *Culex pipiens* and *Anopheles gambiae* (G119S; Weill *et al.*, 2004) as well as in *Culex tritaeniorhynchus* (F331W; Alout *et al.*, 2007). The role of these mutations in reducing the AChE sensitivity to OPs was confirmed by inhibition analysis of mutated AChE expressed in S2 cells (Weill *et al.*, 2003; Oh *et al.*, 2006). Three dimensional modelling showed that the G119 and F331 residues lie within the active ‘gorge’ of the enzyme (Figure 7). The hydrophobic side chain of Glycine is part of the oxyanion hole and participates to the stabilization of the transition state (Figure 2) during the acylation reaction (Zang *et al.*, 2002). The aromatic moiety of F331 is located near the anionic sub-site and it is involved in the proper orienteering of acetylcholine within the catalytic site of AChE (Harel *et al.*, 1993). When mutated AChE forms of *T. urticae* carrying F331W or G119S were expressed in Sf9 cells, a reduction of both sensitivity to OP monocrotophos and the catalytic efficiency of the enzyme were found (Kwon *et al.*, 2012). Although this effect appeared much more evident for F331W mutated AChE, the two substitutions acted synergistically when they were present together. The decrease in catalytic efficiency of modified AChE was consistent with the fitness cost observed in field for mutant mosquitoes (Alout *et al.*, 2008) carry the same mutations even no data are still available for *T. urticae*.

Recently chlorpyrifos resistant strains of the predatory mite *K. aberrans* were described (Tirello *et al.*, 2011). To verify if they had a mutated AChE, with reduced insecticide sensitivity, the cDNA for a paralogous AChE was first cloned in a susceptible strain and its sequence compared to that of a resistant strain. For the cloning step the annotated genome project of the western orchard predatory mite *M. occidentalis* was exploited. Unfortunately more a dozen of AChEs-like sequences were predicted by the curators of the *M. occidentalis* genome using an automated computational analysis based on GNOMON algorithm. Since the GNOMON was trained on the previously deposited AChE sequences in GenBank, and very few of them were from subclass acari, as expected, the predicted AChE-like sequences in the phytoseiidae genomes displayed uncompleted open reading frames or differed in splicing

paths. To identify a possible candidate the AChE sequence from *T. urticae* was used. Once found a putative homologous AChE in *M. occidentalis*, its sequence was used to speed up the cloning of paralogous AChE in *K. aberrans*. When the cDNA sequences from susceptible and resistant strains were compared, only a non synonymous mutation G to A were detected (Figure 6), which introduced a G191S substitution in the protein sequence, corresponding to the G119S as AChE torpedo numbering. To confirm that this mutation was associated to the chlorpyrifos resistant phenotype in *K. aberrans*, it was searched, along with the F331W mutation, in further two susceptible and three resistant strains, by direct sequencing of gene fragments, coding for both amino acid positions. The G191S substitution was present only in resistant strains while it was confirmed that the F331W mutation in *K. aberrans* AChE was not involved in reduced sensitivity to chlorpyrifos. However since the full length cDNA sequencing had been completed just in one resistant strain, it can not be excluded that in the remaining three resistant strains other substitutions might be present beyond the G119S. Weirdly the corresponding amino acid position in the homologous AChE found in the annotated genome of *M. occidentalis* is occupied by a Serine. Unfortunately no information was available concerning the chlorpyrifos sensitivity of *M. occidentalis* strains included in the genome project. Although chlorpyrifos inhibition study of acetylcholinesterases bearing the G119S substitution in *Culex quinquefasciatus* showed a reduced sensitivity of mutated AChE (Liu *et al.*, 2005), *T. urticae* strains with the same AChE genotype displayed *in-vivo* only a moderate resistance to the insecticide. In contrast all examined resistant strain in *K. aberrans* were high resistant to chlorpyrifos (>145,000 fold). So the G119S substitution in cloned *K. aberrans* AChE could be consider as a molecular marker of the resistant phenotype but likely not the unique genetic determinant of resistant phenotype. This finding is in agreement with preliminary backcrossing data between susceptible and resistance strains which seems to indicate a polygenic mode of inheritance of the chlorpyrifos insensitivity (personal communication). Other potential mechanisms linked to target site resistance in subclass acari may consist in amplification of modified AChEs, to compensate the reduction in catalytic efficiency often associated to mutations conferring insecticide insensitivity (Kwon *et al.*, 2010a; Temeyer *et al.*, 2012). Moreover, even if in *T. urticae* genome, only one AChE gene copy was annotated and actively transcribed (Grbic *et al.*, 2011; Anazawa *et al.*, 2003), multiple AChE loci were predicted in *I. scapularis* genome (Van Zee *et al.*, 2007) or cloned and *in-vitro* expressed as functional AChEs, in *R. microplus* (Temeyer *et al.*, 2010). In addition, mutations in different copies of *R. microplus* AChEs are though to contribute with



additive effect to the resistant phenotype, which result under polygenic control (Temeyer *et al.*, 2012). Intriguingly if the cloned KaAChE was used as query sequence in a Blastp research on annotated *M. occidentalis* genome, many putative *in-silico* predicted AChEs were confirmed. Considering just those candidates with conserved functional residues for AChE activity (i.e. forming the catalytic triad and acetylcholine binding pocket) and that received support from transcriptoma analysis (Hoy *et al.*, 2012), probably further six loci for AChE-like sequences are expressed in *M. occidentalis* (GenBank accession numbers XP\_003743019, XP\_003747509, XP\_003739584, XP\_003738701, XP\_003744479, XP\_003745369). Most of these sequences were much more similar to AChE-3 in *R. microplus* (maximum identity range 50-35%) than to AChE in *T. urticae* (maximum identity range < 30%). Actually in *R. microplus* AChE-1 and AChE-3 enzymes with reduced sensitivity to OP-inhibition have been cloned and *in-vitro* expressed, confirming their involvement in OP resistance (Temeyer *et al.*, 2012). Altogether these findings suggest that in predator mites, the target site resistance due to insensitive AChE might resemble the complex pictures found in ticks, with more than one potential target site for insecticide insensitivity. Obviously it cannot be excluded the contribution of metabolic resistance even if higher insensitivities to OPs as that found in the examined *K. aberrans* strain, are mainly associated to target site resistance. In literature very few reports explore the potential metabolic mechanism for chlorpyrifos resistance in acari. In a *T. urticae* strain with an increased insensitivity to chlorpyrifos (91.6 fold) induced by laboratory selection, high level of carboxylesterases activity was found, together with the expression of particular enzyme variants detectable in native PAGE (Ay *et al.*, 2010). In insects a few reports focused on the role played by carboxylesterases as well. Gene amplification of carboxylesterases was described in a 90 fold resistant strain of *C. pipiens* (Buss *et al.*, 2004). Similarly multi-organophosphorus resistance in the saw-toothed grain beetle *Oryzaephilus surinamensis* and in the small brown planthopper, *Laodelphax striatellus* was due to elevated esterase levels (Conyers *et al.*, 1998; Lee *et al.*, 1991; Wang *et al.*, 2010; Zang *et al.*, 2012). Chlorpyrifos selection in the german cockroach, *Blattella germanica* (L.) induced the expression of different carboxylesterases isoforms, instead of an increasing in total esterase activity, along with an augmented level of cytochrome P450 monooxygenases (Scharf *et al.*, 1998). In whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* two molecular mechanisms were proposed: a main decreased in AChE sensitivity, supported by a carboxylesterase mediated hydrolysis or sequestration of the insecticide (Alon *et al.*, 2012; Erdogan *et al.*, 2012). In transcriptoma of *M. occidentalis* (Hoy *et al.*, 2012) seventeen

independent transcripts coding for putative carboxylesterases were detected. Since the AChE cloned in *K. aberrans* was very similar to that annotated in *M. occidentalis*, the same high level of sequence conservation is expected inside the close related carboxylesterase super family. If so the sequence of carboxylesterase transcripts in *M. occidentalis* might be useful to verify whether the over expression of homologous carboxylesterases were involved in chlorpyrifos resistance in *K. aberrans*, as suggested by preliminary results of biochemical analysis performed on the insensitive.

In summary, molecular mechanisms of chlorpyrifos resistance in *K. aberrans* is probably more complex than that described in *T. urticae*. The F331W substitution responsible for high insensitive AChE in *T. urticae* was absent in the homologous gene cloned in resistant strain of *K. aberrans*. However a G119S mutation detected in the same gene appeared strongly associated to the resistant phenotype. According to literature the presence of this mutation can not explain alone the high level of chlorpyrifos resistance detected in *K. aberrans* but it might be useful as molecular marker to test the performance of resistant populations released in field for IPM programs. The cloned AChE sequence, used as probe to query the *M. occidentalis* genome project, confirmed the existence of multi loci AChE genes in predatory mites, contributing to disclose new potential candidates for target site resistance in *K. aberrans*. The sequence similarity found between *K. aberrans* and *M. occidentalis* paralogous AChE suggest that an analogous strategy might be used to explore at molecular level, the role of metabolic detoxifying activity due to carboxylesterases which often supports the target site insensitivity in chlorpyrifos resistance.

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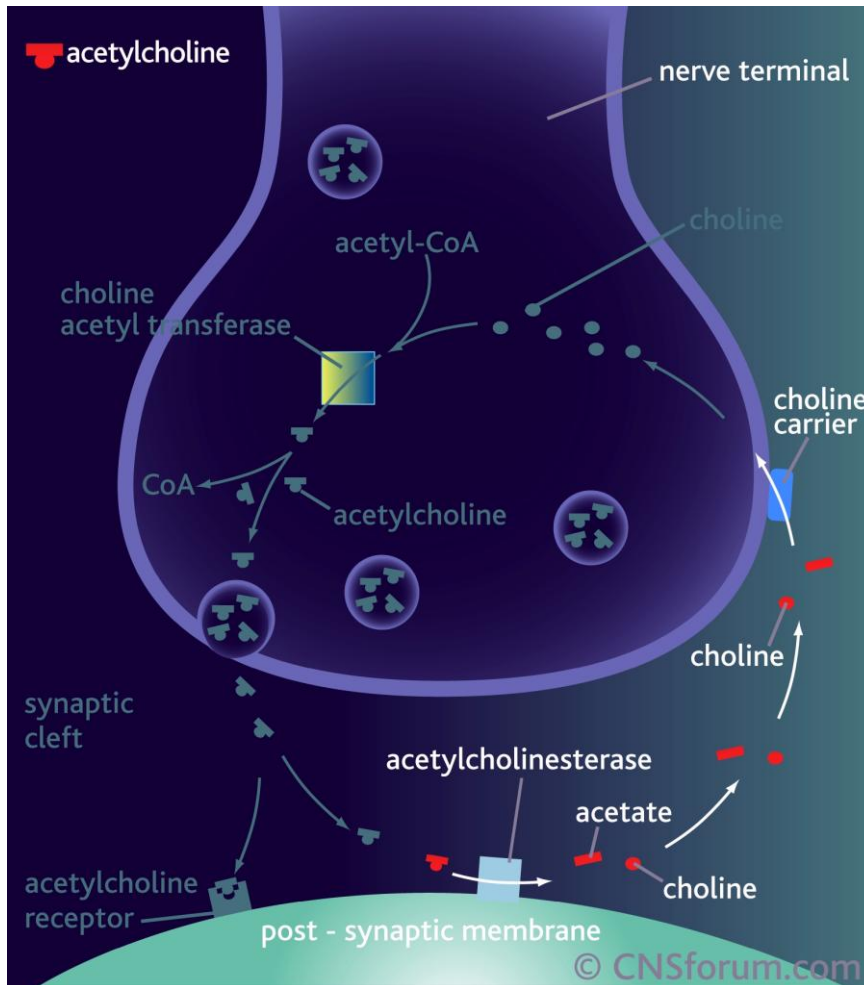


Figure 1 - Acetylcholine cycle. Cholinergic nerve transmission is terminated by the enzyme acetylcholinesterase (AChE). AChE is found both on post-synaptic membrane of cholinergic synapses and in other tissue. Acetylcholine (ACh) binds to AChE and is hydrolysed to acetate and choline. This inactivates the acetylcholine and the nerve impulse is halted. AChE inhibitors (as the chlorpyrifos insecticide) prevent the hydrolysis of ACh, increasing the concentration of the neurotransmitter in the synaptic cleft which in turn results into a neurotoxic effect.



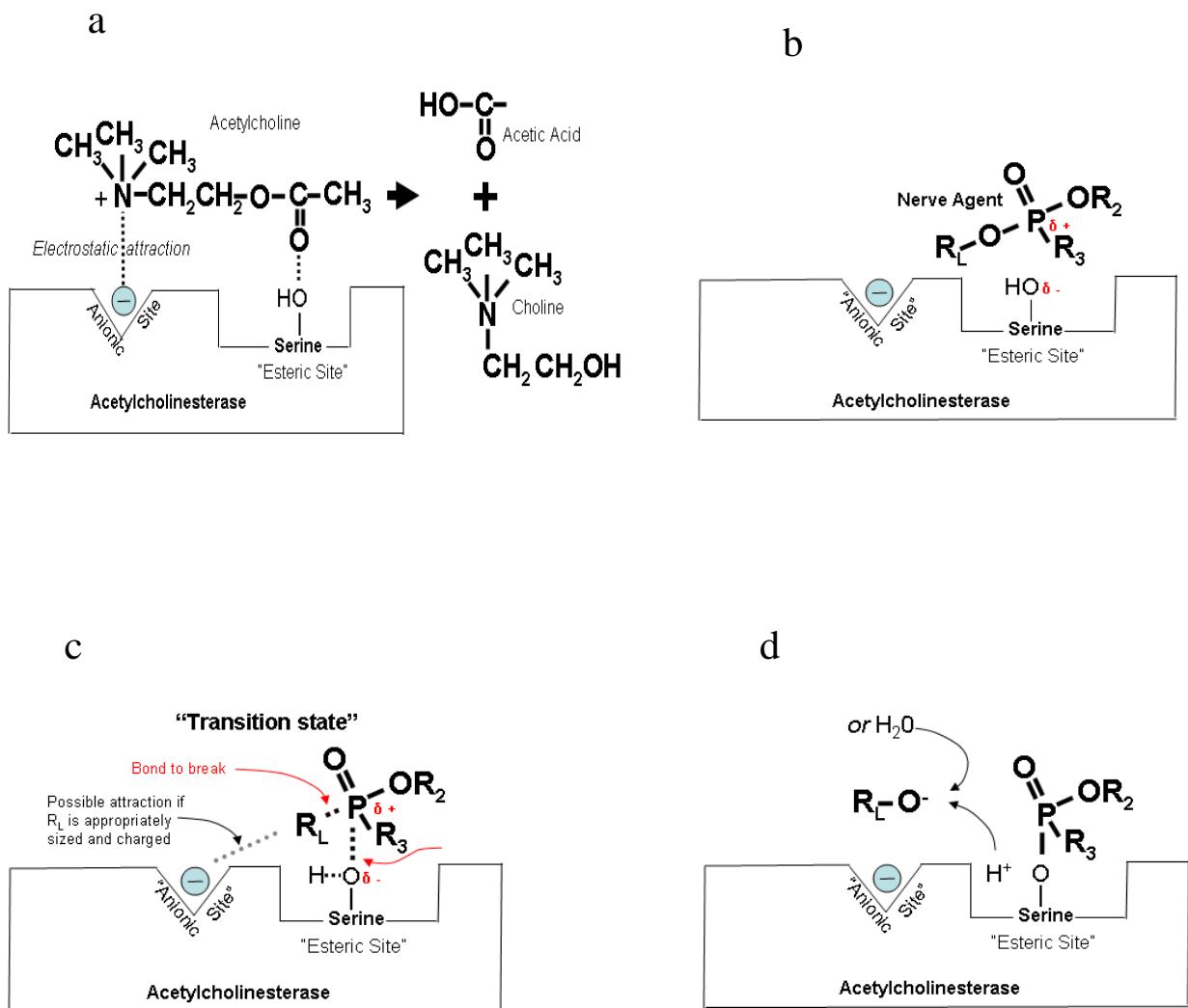


Figure 2 - a) How Acetyl-cholinesterase (AChE) normally works: the positively charged nitrogen in the acetylcholine molecule is attracted to the ionic site on acetylcholinesterase, and hydrolysis is catalyzed at the esteric site to form choline and acetic acid. b) Organophosphate (OP) insecticide as nerve agent. c) Interactions between OP inhibitor and AChE. Partially electropositive phosphorus is attracted to partially electronegative serine. ( $\delta +$  indicates that phosphorus is partially electropositive,  $\delta -$  indicates that oxygen is partially electronegative). d) Phosphorilation of Serine by the OP at the esteric site blocks the hydrolysis of acetylcholine.

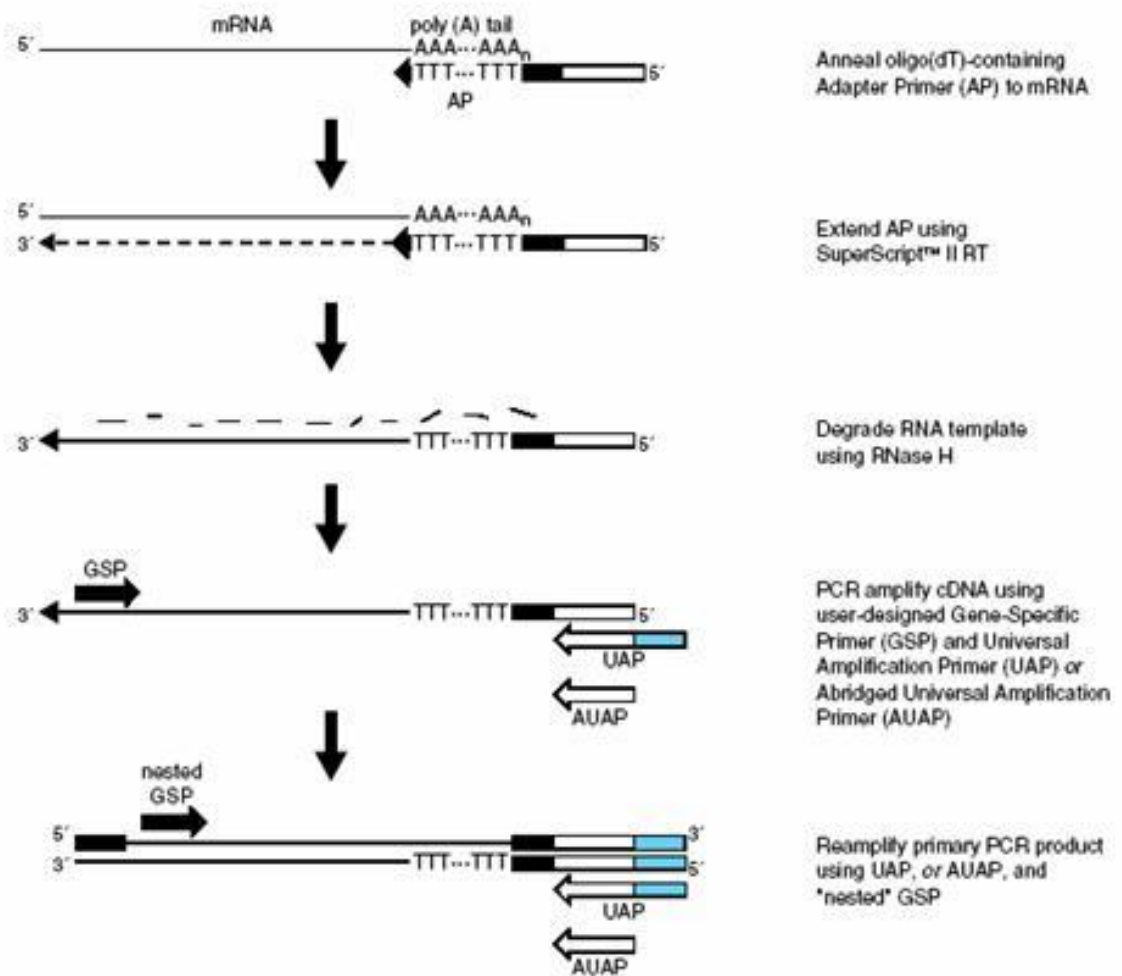


Figure 3A - Rapid amplification of 3' cDNA end (3'RACE) procedure. In the text OligodT containing Adapter stands for polyT-adaptor primer, GSP for KaAChEF4 primer, UAP for Adaptor1, nested GSP for KaAChE-F5, AUAP for Adaptor2.

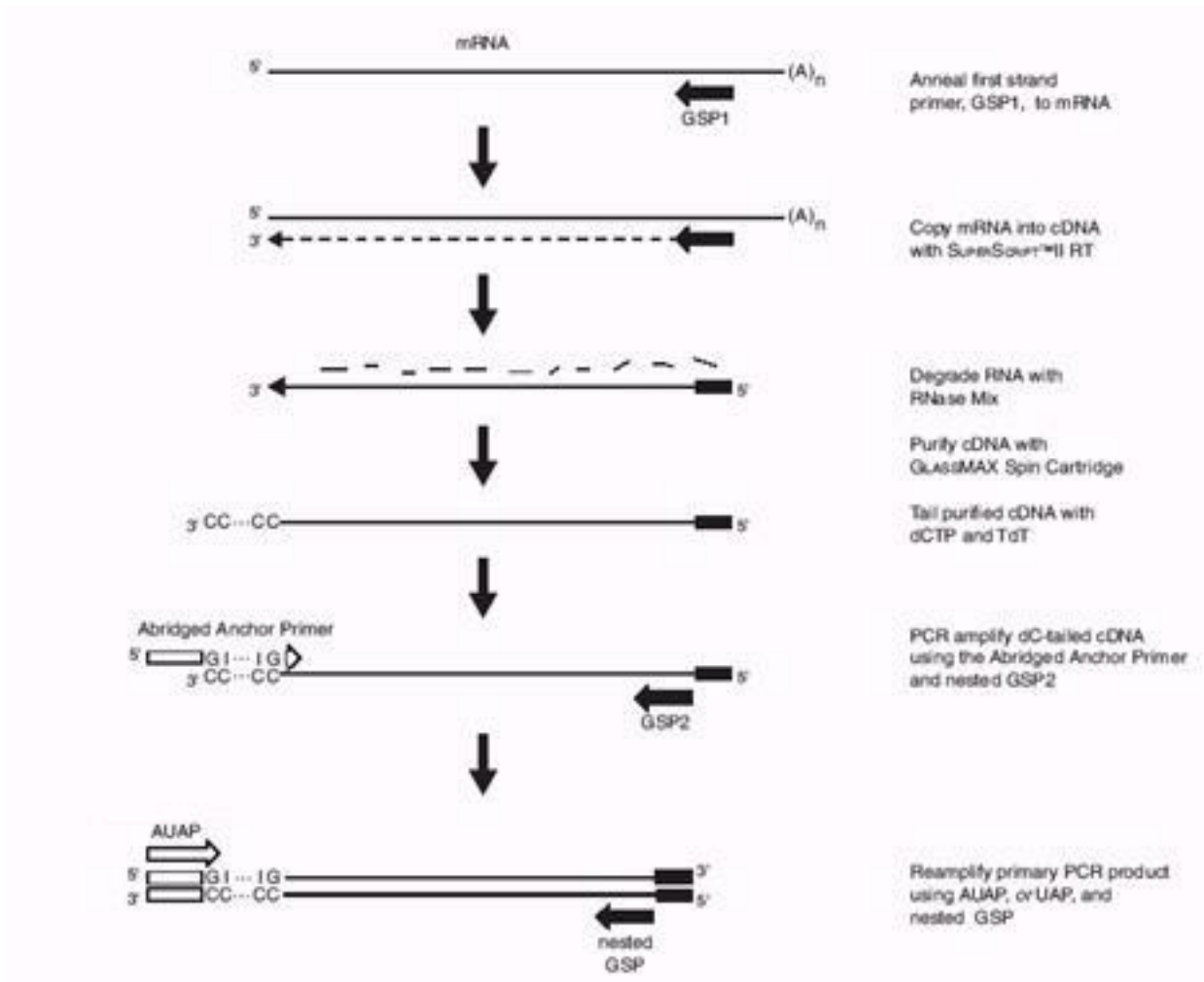


Figure 3B - Rapid amplification of 5' cDNA end (5'RACE) procedure. In the text GSP1 stands for KaAChER4 primer, Abridged Anchor primer for TS-primer, GSP2 for KaAChER5 primer, AUAP for TS-PCR primer and GSP for KaAChER6 primer.

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1 ACACCTTGATAAACTGTGCGCTGTGGCCCCGCGTCCCAGGATACATGTCGTACTCCAGACGTAATTTGCTG 70
-----|-----|-----|-----|-----|-----|-----|
71 ACCCCCGCAAATACGACATCGCTCTCGGAGGACTGGATGATTGGATatgcggttaggagtttcaggcggg 140
1 M R L G V S G G 8
-----|-----|-----|-----|-----|-----|-----|
141 gccagtggttagctaagctagcgtgtgcccgatccatggcggcactgtgcttctgtcgacgcggg 210
9 A S G L A K L A L C R S M A A T V R S L S T R A 32
-----|-----|-----|-----|-----|-----|-----|
211 cgctgtttgcatTTTTgattcattatggctgggtggtcactctatgctcggacgcgtggacgcaagggc 280
33 L F A F L I H L W L V V T L C V G R V D A R A 55
-----|-----|-----|-----|-----|-----|-----|
281 agctcatcttctacaccatcatagacatcgtacggcgggaagcgcgcaatccaaggagatcctcttctg 350
56 A H L L H H H R H R T A G S A Q S Q G D P L L 78
-----|-----|-----|-----|-----|-----|-----|
351 gtccatacaaccaaaggctcccgtccgaggaataaacactgcaggcctccaatgggaaacttgtcgacgcat 420
79 V H T T K G P V R G I T L Q A S N G K L V D A F 102
Int V 178 bp Intron in Metaseiulus occidentalis
-----|-----|-----|-----|-----|-----|-----|
421 tcctcggatttccgtatgCGAAACCACCGTGGGCAAAATGCGATTCGACATCCTGTGCCTATGGACC 490
103 L G I P Y A K P P V G K M R F R H P V P M D P 125
-----|-----|-----|-----|-----|-----|-----|
491 atgggagaagccactcaacgtgacagaacgtcccgccacatgtgtccaagtagttgacacataatttcgac 560
126 W E K P L N V T E R P A T C V Q V V D T Y F D 148
-----|-----|-----|-----|-----|-----|-----|
561 gactttgaggggttcaacaatgtggaatgccaaactaatatgagcgaagactgtttaaacatgcttgttt 630
149 D F E G S T M W N A N T N M S E D C L N M L V W 172
-----|-----|-----|-----|-----|-----|-----|
631 gggttccgCGGCCACGGCGACGAACGCTGCCGCTCTTCTATGGGTTTACGGAGGCGGCTTTTATTCCGG 700
173 V P R P R P T N A A V L L W V Y G G G F Y S G 195
-----|-----|-----|-----|-----|-----|-----|
701 ctgtgccacactggacgtgtacgatgggaaaattctcgcagtggaagaaaacgtaatcgttgtatcattc 770
196 C A T L D V Y D G K I L A S E E N V I V V S F 218
-----|-----|-----|-----|-----|-----|-----|
771 aactatcgggctcggtctctggttctctacctcgatcacgcagacgctccgggcaatgcaggcatga 840
219 N Y R V G S L G F L Y L D H A D A P G N A G M M 242
-----|-----|-----|-----|-----|-----|-----|
841 tggaccaagtgtgctctacgatgggtccaggacaatatacacttgttcggaggcaaccgaacaacgt 910
243 D Q V M A L R W V Q D N I H L F G G N P N N V 265
-----|-----|-----|-----|-----|-----|-----|
911 gactttgttcggggagagcgcaggtgccgtcagcgttgccctaccacctcctctcgcctctctcagggac 980
266 T L F G E S A G A V S V A Y H L L S P L S R D 288
-----|-----|-----|-----|-----|-----|-----|
981 ctattttctcaagccgtgctgcaatccggggagcagctgttccctggggatacaacgagcggcagactg 1050
289 L F S Q A V L Q S G G A T V P W G Y N E R Q T A 312
-----|-----|-----|-----|-----|-----|-----|
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313 M T N G Y K L A E E V K C P T D D V E A T V K 335
m-----|-----|-----|-----|-----|-----|-----|
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336 C L R L Q D P D L L V K S E I F A T G V V D F 358
-----|-----|-----|-----|-----|-----|-----|
1191 tctttcgtccccgtggtggacggcgcattttctcacagagcagaccagaggatacaatgaactcaggaatt 1260
359 S F V P V V D G A F L T E R P E D T M N S G N F 382
-----|-----|-----|-----|-----|-----|-----|
1261 tcaaaaagtcaaaaatactacttgatcgaatcgcgacgaagggacttacttcataatctactatctcac 1330
383 K K C K I L L G S N R D E G T Y F I I Y Y L T 405
G328A F331W
-----|-----|-----|-----|-----|-----|-----|
1331 tcaacttttcaaacgagacgaaaatgtttatctgaccagagaagacttcgctcgcgcgctccaggctctc 1400
406 Q L F K R D E N V Y L T R E D F V D A V Q A L 428

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Int ▽ 194 bp intron in *K. aberrans*

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-----|-----|-----|-----|-----|-----|-----|
1401 agtccttttacctcgcaggtggtgaacgaagccatcatttttgagtacacggactggttgaaccctgacg 1470
429 S P F T S Q V V N E A I I F E Y T D W L N P D D 452
-----|-----|-----|-----|-----|-----|
1471 atccgatcaaaaatcgagatgccgctcgacaagattggtggcgattactacttcacatgcccggtaatcga 1540
453 P I K N R D A V D K I V G D Y Y F T C P V I D 475
-----|-----|-----|-----|-----|-----|
1541 tacggcacactattattcatcggtggactcgatgtctacatgtactactacgtgtaccgctcgtctcag 1610
476 T A H Y Y S S A G L D V Y M Y Y Y V Y R S S Q 498
-----|-----|-----|-----|-----|-----|
1611 aataaatggccggagtggtggagtgatccacgccgacgagattgcttacgtcttcggcgagccgctca 1680
499 N K W P E W M G V I H A D E I A Y V F G E P L N 522
-----|-----|-----|-----|-----|-----|
1681 accaaacatggtcttaccgccaagacgaacagatggttagtcgccgcattatgcggtactgggccaattt 1750
523 Q T W S Y R Q D E Q M F S R R I M R Y W A N F 545
-----|-----|-----|-----|-----|-----|
1751 tgcgcgaatgggaaacccgagcctgaaccccgacggaactgggagaaaacatattggccggcgcacact 1820
546 A R M G N P S L N P D G N W E K T Y W P A H T 568
-----|-----|-----|-----|-----|-----|
1821 gcgttcggaaggagtctcctcatcctcgacgtcaactcgacgcaagttggatacgggaaccgagcgaac 1890
569 A F G K E F L I L D V N S T Q V G Y G N R A K H 592
-----|-----|-----|-----|-----|-----|
1891 attgcgattctggaagaatttccttccaaacctcatcgctatcgggcaacaacacaaaagaagccga 1960
593 C A F W K N F L P N L I A L S G N N T N K A E 615
-----|-----|-----|-----|-----|-----|
1961 ggaagctgcagagacggagcgagttcgacagctcgtcgtccatcatgctcttatgctcattggcggca 2030
616 E G C R D G A S S Q S S S S I M L L C S L A A 638
-----|-----|-----|-----|-----|-----|
2031 agcatagtcgtcacggccgtattctgtcacaaccaccggcgacagcagcgtagCTTCAGCTGATAACAG 2100
639 S I V V T G R I L S Q P P A T A A * 655
-----|-----|-----|-----|-----|-----|
2101 GGTCTACAATTCTGCCATGACACCGACAAGTCAACATGTAATCCAAACATGTAACCACCTCGA 2170
-----|-----|-----|-----|-----|-----|
2171 AGTCGTGCTCCAACCTGAAGGAGCGAACTAGGATTGTTGCTGAAAATGTTACAACCACCACAATCGGCAAA 2240
-----|-----|-----|-----|-----|-----|
2241 AGGCAGCACCGGAAGCAACGGCAGCAGCCGCTGCAAGTGAAAGCTGAAGCTCGCTTAAACGGAGTGTGAC 2310
-----|-----|-----|-----|-----|-----|
2311 CGAAAGAACAAGCCCACCC 2329

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Figure 4 - Alignment of acetylcholinesterase coding cDNA and protein sequence, cloned in susceptible strain of *K. aberrans*. The asterisk indicates the stop codon. Residues forming the functional domains are in different colours: in green cysteines forming intramolecular disulphide bonds, in orange amino acid residues of the catalytic triad, in violet the anionic subsite, in red the residues of the oxyanion holes and in pink the acyl pocket residues. G119S substitution found in AChE sequence both in resistant strains of *T. urticae* and *K. aberrans* is underlined and in red colour. In blue G328A and in green F331W substitutions found in insensitive *T. urticae* AChE. The solid inverted triangle shows the position of the intron amplified during the genomic screening of F331W and G328A substitutions.

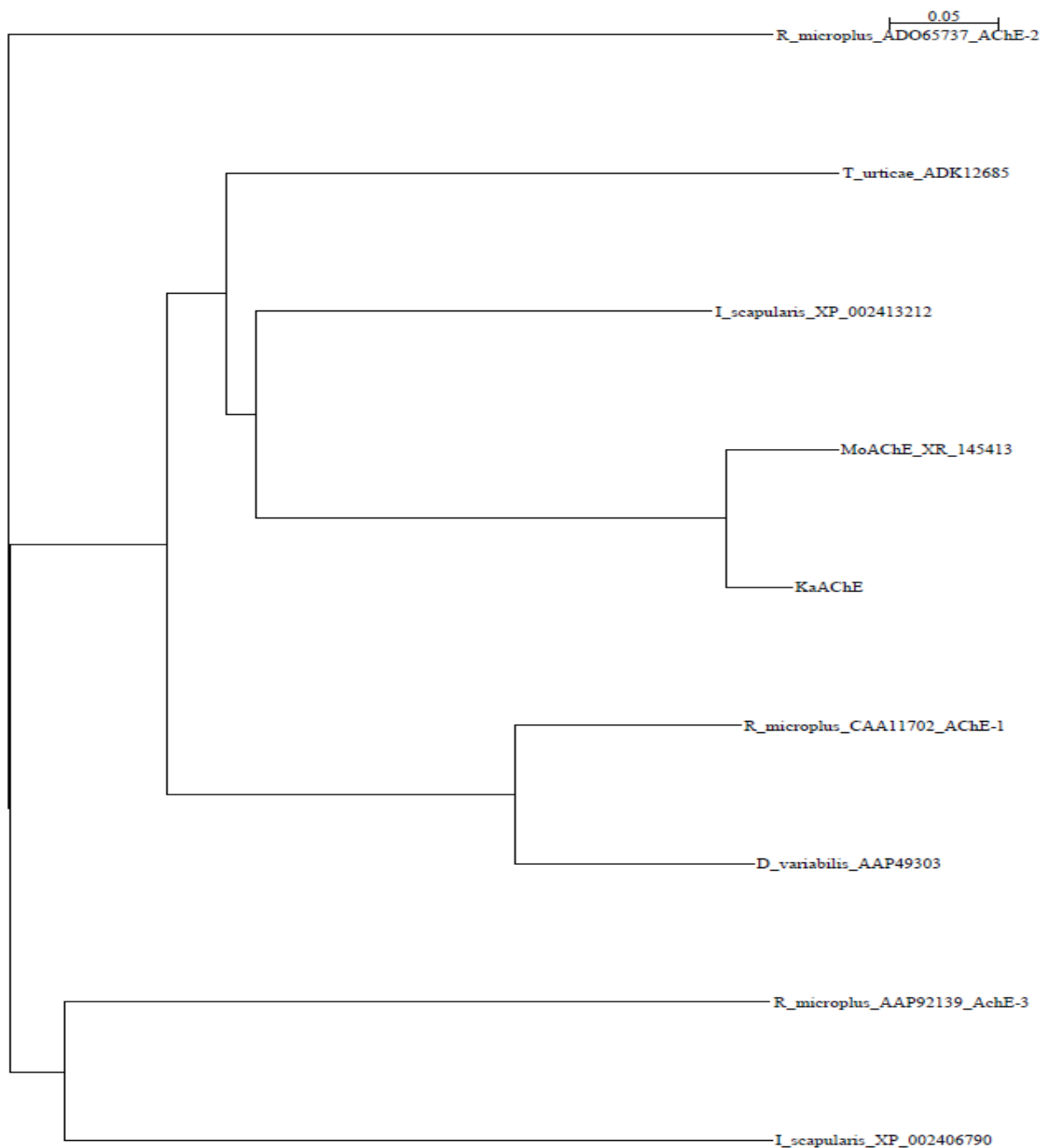


Figure 5 - Rooted phylogenetic tree of representative AChEs found in Acari subclass, by neighbour joining method, using AChE-2 cloned in *R. microplus* (GenBank number ADO65737) as outgroup. The scale bar represents 5 percent divergence. For tree construction used AChE annotated in genome project of *Ixodes scapularis* (XP\_002413212, XP\_002406790) and *Metaseiulus occidentalis* (XR\_145413) or cloned in *Rhipicephalus microplus* (CAA11702, AAP92139), in *Dermacentor variabilis* (AAP49303) and in *Kampimodromus aberrans* (this study).

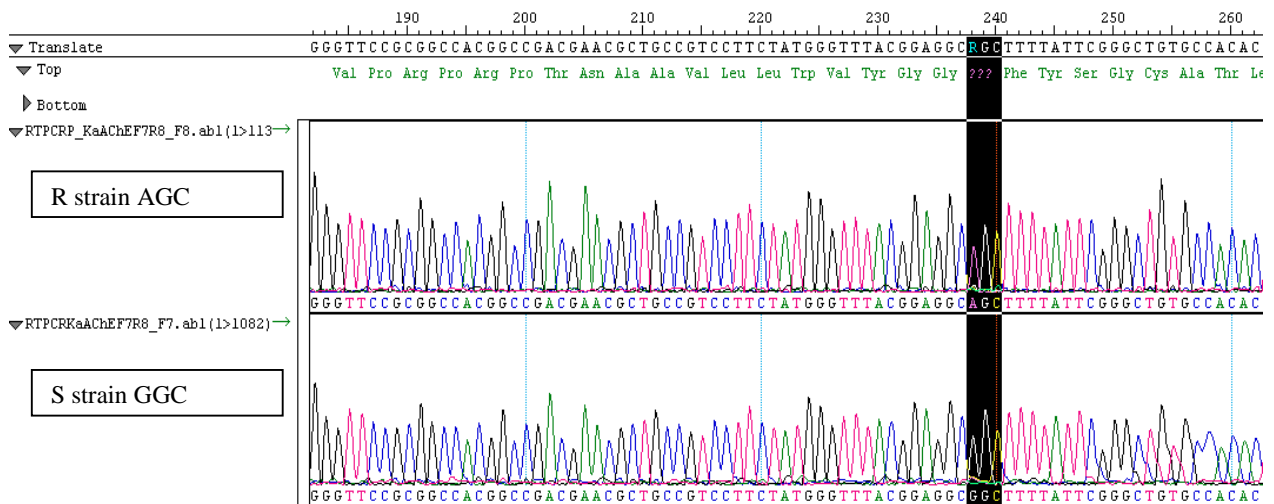


Figure 6 - The AChE genotype with the G-to-A single nucleotide polymorphism for G119S substitution (AChE *Torpedo californica* numbering) in susceptible (S) and in resistant (R) strains of *K. aberrans*.

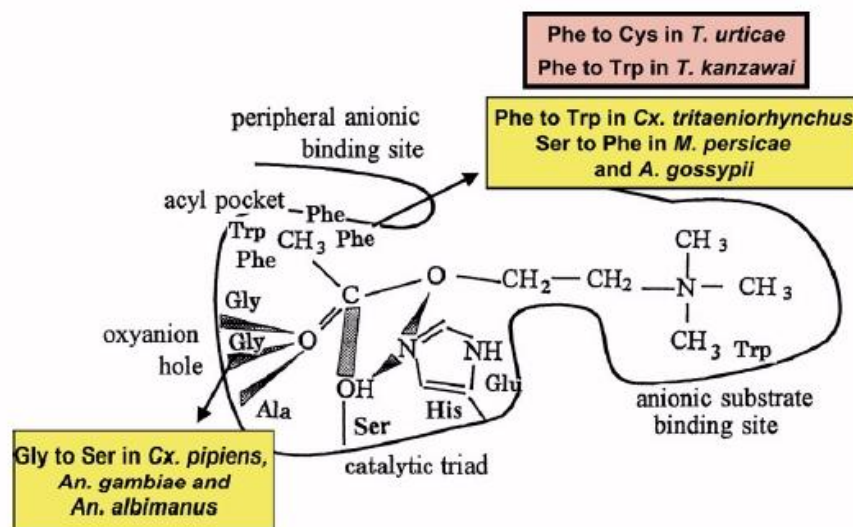


Figure 7 - Schematic picture of AChE active site with amino acid substitutions which impair the catalytic function (Aiki *et al.*, 2005)

**Table 1 - Primer sequences**

	Forward primer		Reverse primer	
<i>Degenerate primers</i>		5' → 3'		5' → 3'
	KaAChEF1d	GGNATHCCNTAYGCNAARCC	KaAChER1d	ACCCANATRCCZCCNAGZAAK
	KaAChEF2d	CCNTAYGCZAARCCNCC	KaAChER2d	ACCCANATRCCZCCNTCZAAK
<i>3' cDNA extension</i>				
	KaAChEF3	GTTGAACCCTGACGATCCGATCAA	KaAChER3	AGGAAGTTCTTCCAAAACGCGCA
<i>3' RACE</i>				
			oligdTadapter	ACAGCAGGTCAGTCAAGCAGTAGCA GCAGTTCGATAAGCGGCCCGCCATGG AT12 DN
	KaAChEF4	AGGTGGTGAACGAAGCCATCAT	Adapter1	ACAGCAGGTCAGTCAAG
	KaAChEF5	TTGAGTACACGGACTGGTTGAACCCT	Adapter2	AGCAGTAGCAGCAGTTCGATA
<i>5' RACE</i>				
			KaAChER4	CTCCGTAAACCCATAGAAGGA
	TS-Primer	CACCATCGATGTCGACACGCGTCGGGIGGIG	KaAChER5	CCACATTGTTGAACCCTCAAAGTCG



	TS-Pr	CATCGATGTCGACACGCGTC	KaAChER6	TGTTGAACCCTCAAAGTCGTCG
<i>cDNA sequencing</i>				
	KaAChEF6	ACCTTGATAAACTGTCGCTGTGGC	KaAChER7	AAAGTCACGTTGTTCGGGTTGCCT
	KaAChEF7	CAATGCAGGCATGATGGACCAAGT	KaAChER8	AGGAAATTCTTCCAGAATGCGCA
	KaAChEF8	GTTGAACCCTGACGATCCGATCAA	KaAChER9	TCCTAGTTCGCTCCTTCAGTTGGA
	KaAChEF9	AAATGCGATTTTCGACATCCTGTGCC	KaAChER10	CCAACAATCTTGTCGACGGCATCT
	KaAChEF10	GCGCTATCGGGCAACAACACAAACA		
<i>G119S screening</i>				
	KaAChEF11	AATGCGATTTTCGACATCCTGTGCC	KaAChER11	AAAGTCCGTTGTTCGGGTTGCC
<i>F331W screening</i>	KaAChEF12	AAACTCGCGGAGGAAGTCAAGTGT	KaAChER12	CCAACAATCTTGTCGACGGCATCT

## **Conclusions**



The effects of pesticides on predatory mites occurring in apple orchards have been studied following a multi-step approach in a number of experiments planned at field, semi-field and laboratory levels. Among predatory mites, we selected *Kampimodromus aberrans*, a species successfully used in European vineyards but poorly considered in IPM tactics in apple orchards. The main scopes of this study were to assess: a) the potential of *K. aberrans* populations to establish and play a key role in apple orchards, c) the effect of interspecific competition in this context; b) the impact of pesticides in preventing the activity of predatory mites as biocontrol agents, c) the role of alternative foods in mitigating the pesticide effects on predatory mites, d) the mechanisms evolved by *K. aberrans* pesticide resistant strains to reduce the impact of pesticides.

Releases of *K. aberrans* were made in conventional and organic apple cultivars. Predatory mites were significantly higher in released plots as compared to control plots and *K. aberrans* populations were higher in organic compared to conventional orchards. However, releases were fully successful only in some organic orchards and especially on cultivars having favorable leaf morphological features for predatory mites. Populations of the native predatory mite *Amblyseius andersoni* were lower in released than in control plots. We can suggest that the use of non-selective pesticides affected *K. aberrans* colonization in conventional apple orchards, and also multiple application of spinosad were associated with a significant reduction in *K. aberrans* populations in organic orchards. Therefore, pesticides, host plant features and interspecific competition were key factors in the establishment of *K. aberrans* in apple orchards.

Interspecific competition proved to be an important factor affecting the structure of mite communities in orchards. Four predatory mites common in European apple orchards (*A. andersoni*, *T. pyri*, *K. aberrans* and *P. finitimus*) were tested in reciprocal predation experiments in which females were fed with heterospecific larvae. All predatory mite species were able to survive and oviposit on heterospecific larvae. In terms of predation rate and fecundity, *A. andersoni* seems to be advantaged over *T. pyri*, *T. pyri* over *K. aberrans*, and *A. andersoni*, *K. aberrans* and *T. pyri* over *P. finitimus*. Most of these results were expected to be influenced by relative aggressiveness that is partly associated to body size. However, *A. andersoni* females exhibited a higher performance on *T. pyri* than on the slightly smaller *K. aberrans*. The performance of *A. andersoni*, *K. aberrans* and *T. pyri* in terms of predation rate and fecundity proved to be better of *P. finitimus* larvae than on other prey. The low prey consumption and fecundity of the latter suggest that is disadvantaged in interspecific

predation. The comparison of performance exhibited by predatory mites on specific prey species additional information. *Amblyseius andersoni* confirmed to be the most aggressive among these species but its voracity did not imply higher fecundity rates; therefore this predator was the least efficient in converting prey food into egg biomass. Regarding the last two parameters *T. pyri* (and to a lesser extent *K. aberrans*) was superior to *A. andersoni*. Some interesting aspects emerged in the comparison between *K. aberrans* and *A. andersoni*: *K. aberrans* laid more eggs when *P. finitimus* was offered as prey and the fecundity of *A. andersoni* and *K. aberrans* did not differ on a diet based on *T. pyri* larvae. In both cases, prey conversion rate was higher for *K. aberrans* than for *A. andersoni*. This parameter could be considered as an indicator of the capacity to survive when prey is diminishing. The comparison between pollen and prey diets confirmed the positive effect of pollen on the fecundity of all four predatory mite species. Fecundity was higher on pollen than on predatory mite larvae.

Knowledge of pesticide side-effects is fundamental for maintaining the population of beneficial mites and also to prevent risks of pest outbreaks. The present study shed light on lethal and sub-lethal effects of pesticides frequently used in orchards. In field and laboratory experiments, etofenprox, tau-fluvalinate and spinosad proved to be harmful to *K. aberrans* and induced spider mite outbreak. Laboratory studies evidenced sub-lethal effects of pesticides (e.g., neonicotinoids) with potential implications for IPM. The ecological significance and practical consequences of a high escape rate should be investigated more in depth.

The reduction of pesticide side-effects on non-target arthropods is a necessary requisite for conservation biological control. We tried to assess if increasing alternative food availability can mitigate the effect of pesticides on natural enemies and promote their persistence in agro-ecosystems. In laboratory experiments, the provision of a high amount of pollen and the increase of pollen application frequency augmented predatory mite fecundity. These results confirmed that predatory mite fecundity depends on food amount and stress the importance of the freshness of food provided by frequent pollen application. The effect of pollen application frequency was independent from the pollen amount provided. Potted plant experiments corroborated the positive effect of pollen on predatory mite population: pollen application induced higher predatory mite population levels. These results confirmed the importance of pollen as food source for *K. aberrans*. We showed the detrimental effects of spinosad and chlorpyrifos on *K. aberrans*. Both insecticides reduced the survival of this predator, but the

mortality caused by chlorpyrifos was less dramatic than that by spinosad. Fecundity was also reduced by insecticides. No eggs were laid by the few females that survived spinosad, while chlorpyrifos caused a significant reduction in fecundity. Potted plant experiments confirmed the detrimental effects of insecticides, but with a lower magnitude of the effects than that seen in the laboratory. The results obtained highlighted the role of pollen in influencing the impact of pesticides on predatory mites. In laboratory trials the increase in frequency of pollen application compensated for the negative effect of chlorpyrifos on fecundity. This effect was not observed with spinosad. Apart from the effect of pollen amount, the availability of fresh pollen appears to be of particular importance. Having a higher nutritional value, fresh pollen compensated for the insecticide effects and promoted the reproduction of predatory mites. The provision of uncontaminated fresh pollen decreased the exposure to chlorpyrifos with a reduction in sub-lethal effects. This effect was also clear on potted plants. At the end of the experiment, the number of predatory mites on pollen treated plants was similar between control and chlorpyrifos.

Pesticide resistance mechanisms in *K. aberrans* have been poorly explored. Our study on target site resistance to chlorpyrifos in insensitive Acetylcholinesterase (AChE) revealed that AChE mutations conferring chlorpyrifos resistance to *T. urticae* AChE are not present in AChE *K. aberrans*. One mutation G119S substitution is associated to three resistant phenotypes of *K. aberrans*. This mutation could reduce the AChE sensitivity to chlorpyrifos as occurs in other species. This seems important because this could be in linkage with loci responsible for the chlorpyrifos insensitivity, acting as molecular marker of the resistant phenotype.

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