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**Molecular analysis of predation by
anthocorid bugs on the pear psyllid
Cacopsylla pyri (Homoptera, Psyllidae).**

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Acknowledgements

This master thesis was one of two theses written as a part of NIBIO's project on integrated pest management of apple aphids and pear psyllids ("Integrert bekjempelse av eplebladlus og pæresuger"), funded by the Norwegian Research Council. The theses also received funding from "Foregangsfylket for økologisk frukt og bær". By dividing the work in two, the idea was that one thesis would have a more ecological focus, with emphasis on temporal and spatial distributions of anthocorid bugs in relation to pear psyllids, whereas the other, this thesis, would investigate anthocorid predation using molecular methods. As I have virtually no background in molecular biology, this task seemed quite daunting, but as the topic was both current and extremely interesting, I tried to acquire as much knowledge as I could in a short amount of time. It has been a lot of hard work, probably more than anyone would have thought, and many rounds of trying and failing, but I can definitely say that I have learned a lot. However, I would never have been able to complete my thesis in time if it wasn't for this bunch of helpful and knowledgeable people.

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Abstract

Understanding the trophic interactions between prey and its predators is vital for both population ecology and integrated pest management (IPM). In IPM, knowledge of how the trophic interactions in an ecosystem affect prey densities are exploited to improve pest management strategies. In Norwegian pear orchards, excessive use of non-specific pesticides has led the pear psyllid *Cacopsylla pyri* to become a major pest. Because of a renewed focus on pear production in Norway, alternative pest management strategies are needed. The predatory bugs *Anthocoris nemorum* and *A. nemoralis* are two of the most abundant predators in fruit orchards, and are considered a vital part of the natural control of pome fruit pest species. Both species are considered generalist predators, though *A. nemoralis* has been shown to prefer psyllids over other prey.

In order to assess the importance of *A. nemorum* and *A. nemoralis* as beneficial predators in Norwegian pear orchards, field samples were collected from a commercial pear orchard in Hardanger. Molecular gut-content analysis was performed in the laboratory to verify presence of prey DNA, using PCR including previously designed primers by Agustí et al. (2003b). To study the functional response of anthocorid bugs to *C. pyri*, the predation rate (defined as the proportion of psyllid-positive bugs) was compared to field prey densities. In addition, feeding studies were conducted in the laboratory to determine how long *C. pyri* DNA is detectable in the guts of *A. nemorum* and *A. nemoralis* under Norwegian field conditions.

The primer set Cp2F/Cp7R, reported to be specific for *Cacopsylla pyricola* and *C. pyri*, failed to amplify *C. pyri* DNA in this study. However, a second primer set, Cp3F/Cp6R, did successfully amplify all Psyllidae species tested, and showed no cross-amplification of non-target organisms. Detection half-lives obtained in this study were much longer than expected, and possible reasons are discussed. Using the primer set Cp3F/Cp6R, a high predation rate on psyllids was detected, ranging from 82-92%, supporting the assumption that anthocorid bugs are important beneficial predators in fruit orchards. In addition, the relatively high number of anthocorids present in the field was surprising. *C. pyri* constituted 89% of prey available, indicating that *C. pyri* is an important part of *A. nemorum* and *A. nemoralis* diets. Thus, the results obtained in this study suggest the importance of generalist predators in pest control. Lastly, *A. nemorum* predation on psyllids was found to be negatively related to the presence of the ant *Lasius niger*.

Table of Contents

Abstract	I
1. Introduction	1
2. Materials and methods.....	7
2.1 Study site	7
2.2 Field collection	8
2.3 Feeding experiments	9
2.4 DNA extractions.....	10
2.5 Primers	11
2.6 PCR amplification and optimization	11
2.7 Primer specificity test.....	12
2.8 Verification of DNA results	12
2.9 Statistical analysis	12
3. Results	14
3.1 Primer success and specificity.....	14
3.2 Feeding experiments	15
3.3 Arthropods found in the field collection	17
3.4 Field predation.....	18
4. Discussion.....	21
4.1 Primer success and specificity.....	21
4.2 Feeding experiments	23
4.3 Field predation.....	26
4.5 Future focus.....	30
5. Conclusion.....	32
References	33

1. Introduction

Understanding trophic interactions between an individual, its prey, predators, conspecifics and abiotic habitat is a fundamental part of ecology, as these relationships drive population dynamics, species richness and ecosystem stability (Gullan & Cranston 2010; Sih et al. 1985; Symondson 2002). Identifying and quantifying these relationships is therefore an essential part of answering many ecological questions (Juen & Traugott 2006).

In integrated pest management (IPM), knowledge of how a pest species is affected by the trophic interactions in its ecosystem is exploited to develop and improve successful management strategies (Symondson 2002). IPM is an ecosystem approach, where the goal is to keep crop pests below the economic injury level, while minimizing the use of harmful pesticides (FAO 1967; Stern et al. 1959; van den Bosch & Stern 1962). This is done by combining several management methods, such as biological control, use of pheromones, mechanical and physical control, biotechnology, chemical control and appropriate cultural practices (Gómez Polo 2014; Hoy & Herzog 1985). Biological control is usually a major part of IPM, as the abundance and distribution of a species is strongly influenced by the occurrence of natural enemies (e.g. predators, antagonists, parasites/parasitoids and pathogens) (Gullan & Cranston 2010; Stern et al. 1959). As such, mass-releasing natural enemies and/or creating attractive environments for natural enemies (by planting host plants or avoiding harmful pesticides etc.), can increase the natural predation of a pest species, keeping pests at a tolerable level (Hågvar 2004; Sigsgaard et al. 2006). However, thorough knowledge of the ecology and lifecycle of both pest and predator species, as well as the ecological communities in which they belong, is crucial before implementing biological control as a part of the pest management in a certain crop or area.

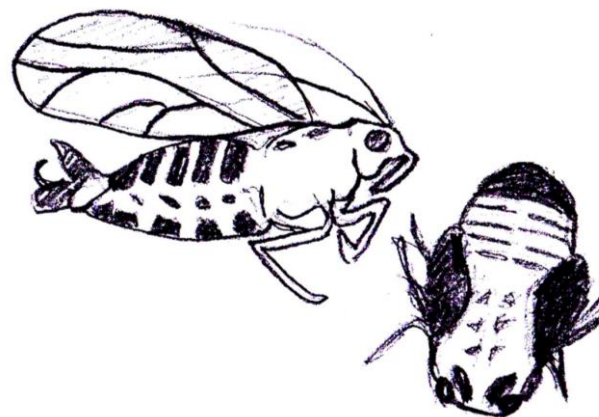


Figure 1: *Cacopsylla pyri* adult (left) and 4-5th instar nymph (right). Length adult: ~3 mm, length nymph: 1,5-2mm. Drawing by Ida Gundersen.

In Europe, IPM was first developed and used by fruit entomologists (Damos et al. 2015), as a response to failing pesticides and an increased concern for the health and environmental risks associated with chemical control. In many cases, the application of pesticides seemed to increase orchard pest populations (Civolani 2012; Solomon et al. 2000), most likely because of the rapid development of pesticide resistance in pests (Buès et al. 1999; 2003; Burts et al. 1989; Harries & Burts 1965) and the adverse effects of pesticides on natural enemies (Croft 1990; Desneux et al. 2007; Moreby et al. 1997; Pimentel 2005). This is evident in European pear production, where excessive use of non-selective pesticides against pear psyllids has effectively decreased the pest-control, causing pear psyllids to become a major pest in pear orchards across Europe (Civolani 2012; Eler 2004; Hodkinson 1984; Solomon et al. 1989; Trapman & Blommers 1992).

In Norwegian pear orchards, the pear psyllid *Cacopsylla pyri* (Homoptera, Psyllidae)(Figure 1) is considered one of the most damaging pest species, and is found all over the country where pear is grown (Edland 2004; Jaastad & Børve 2009). The nymphs cause the most damage, feeding on the sap of flowers, fruits and shoots, producing thick coatings of honeydew. Honeydew marks fruits and facilitates fungal infections, especially by sooty mold, rendering fruit inedible and hindering photosynthesis. Heavy attacks can lead to deformed shoots and fruits, causing fruit to fall off early and negatively affecting next year's crop yield. *C. pyri* has two generations per year in Norway (though a third generation has been observed during warmer summers) (Figure 2), where the second generation causes the most damage. Adults overwinter in cracks in the tree bark of fruit trees, and emerge in early spring, producing eggs as soon as the temperature exceeds 10°C.

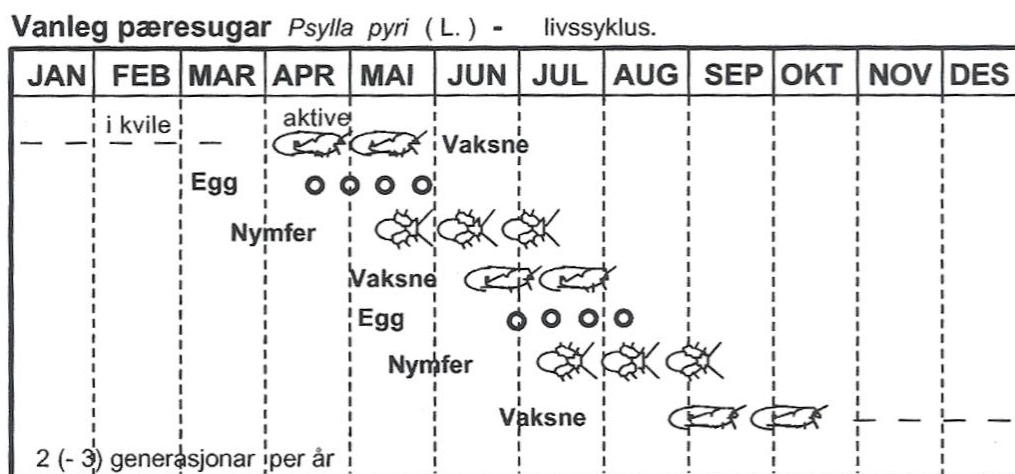


Figure 2: The lifecycle of *Cacopsylla pyri* in Norway. Adults overwinter in cracks in the bark of fruit trees and emerge in march. As soon as the temperature reaches 10°C (for two consecutive days) ovipositioning starts. *C. pyri* has two generations in Norway, where the second generation of nymphs is the most damaging for pear production. Vaksne = adults, Nymfer = nymphs. Taken from Edland (2004).

Because of a renewed focus on Norwegian pear production (Landbruks- og matdepartementet 2011), a better understanding of pear psyllid control, both chemical and biological, is needed. Currently, pesticides are the most common method of controlling pear psyllid populations in Norwegian pear orchards, though there is a recurring problem of increased pesticide resistance (Sundbye et al. 2014). Similar results as what is reported in Europe and North America is also seen here, with increasing pest-populations after application of pesticides (pers. comm., J. Lutro, pear grower hosting the study 2015)(Jaastad & Børve 2009). Thus, information on natural predators of *C. pyri* and factors affecting predator-prey interactions in pear orchards is necessary in order to implement more sustainable pest management.

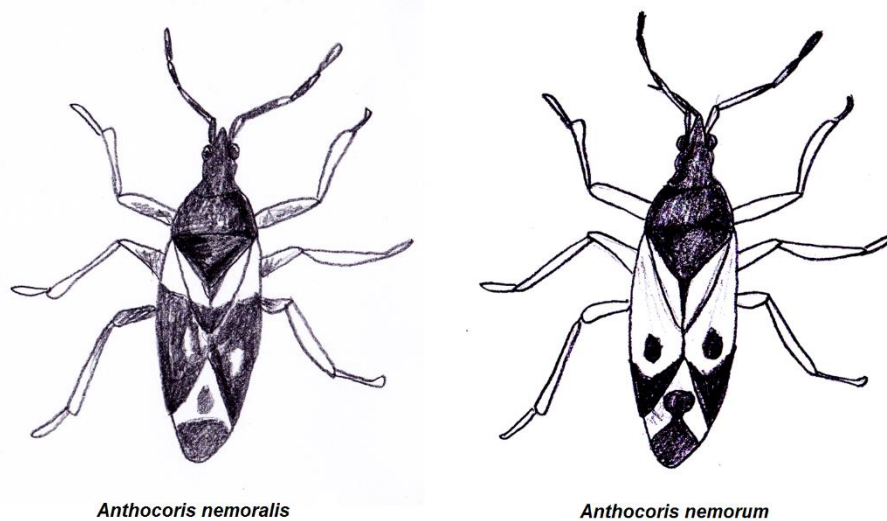


Figure 3: *Anthocoris nemoralis* and *A. nemorum*. Length: 3-4mm. Body of *A. nemorum* is slightly larger/broader than *A. nemoralis*. Drawing by Ida Gundersen.

Anthocoris nemorum and *A. nemoralis* (Heteroptera, Anthocoridae) (Figure 3) are two of the most abundant predators in fruit orchards in Europe (Hill 1957; Solomon et al. 1989; 2000), and have been shown to be important generalist predators in pear orchards. Both field and laboratory experiments indicate that anthocorid bugs are attracted to psyllid-infested pear trees, with increasing attraction to higher psyllid densities (Anderson 1962a; Drukker & Sabelis 1990; Drukker et al. 1995; 2000; Scutareanu et al. 1997). Though not as common in Norway, *A. nemoralis* in particular has been the focus of many studies in Europe, as it shows a clear preference for psyllids, especially *C. pyri* (Anderson 1962b; Dempster 1963; Sigsgaard 2010; Solomon et al. 2000). In Norway, *A. nemorum* has long been considered one of the most important predators in pear orchards (Edland 2004; Sundbye et al. 2014; Våge 1991), feeding on several pest species such as psyllids, aphids, mites, thrips and lepidopteran eggs and small

1. Introduction

larvae (Anderson 1962b; Collyer 1967; Hill 1957). The two *Anthocoris* species overwinter as adults, and are active as soon as weather permits (Figure 4), at which point they aggregate to *Salix spp.* and nettle, before later immigrating into orchards in search of oviposition sites.

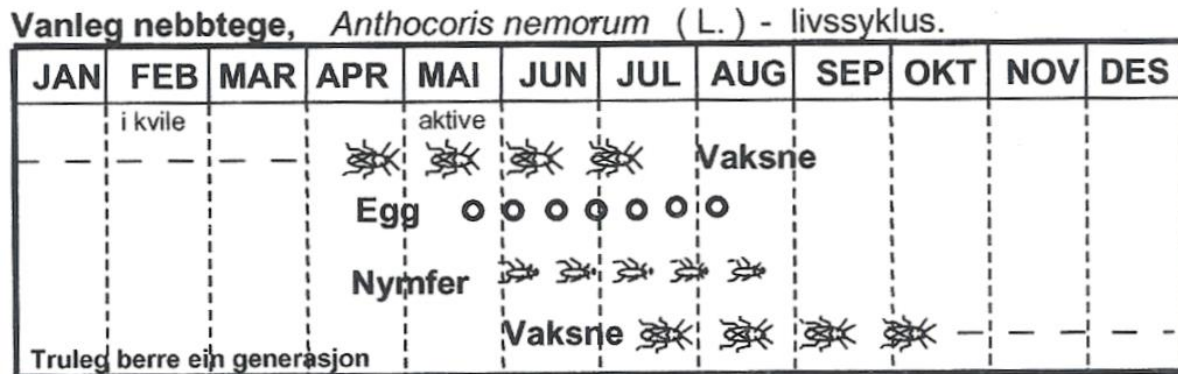


Figure 4: The lifecycle of *Anthocoris nemorum* in Norway. Adults overwinter under leaves and loos bark on the ground. They become active in early spring, at which point they aggregate to *Salix spp.* and nettle, before later immigrating into orchards to lay eggs. Vaksne = adults, Nymfer = nymphs. Taken from Edland (2004)

Investigating predator responses to pest populations, and how these responses are affected by other trophic interactions, is important when quantifying the predation impact of a predator on a pest species. Because generalist predators consume a variety of species, they can switch among different prey depending on prey abundance and/or profitability, giving them the advantage of not being dependent on adequate populations of one particular prey species to survive (Symondson et al. 2002). According to optimal foraging theory, a predator will choose prey based on an optimal trade-off between the cost of foraging and the benefit of energy consumed (Charnov 1976). Generalists can show preference towards certain prey, choosing to mainly feed on this when prey densities are above a certain level. Thus, although generalist predators are unlikely to impact pest species once their population increases exponentially, they may have a combined greater role than individual specialists (Murdoch et al. 1985; Symondson et al. 2002). In addition, as early predation of pest species has been shown to delay, or even prevent pest outbreaks, generalist predators may exert important control early in the season, keeping pest populations at a tolerable level until more specialized predators arrive in the field. Analysis of predation in the field where multiple prey species are available, can therefore yield important information on the potential of generalist predators as biocontrol agents (Hatteland et al. 2011; Murdoch et al. 1985).

However, identifying predator-prey interactions in the field can be very difficult and time consuming, especially in arthropods where the individuals in question are small, cryptic and

highly mobile. Attempting to observe these interactions will also most likely require us to disturb the system, by for example removing/thinning vegetation, thus affecting the recorded results (Symondson 2002). When studying predation in the laboratory, we are unable to perfectly recreate such factors as vegetation, micro climate and the ratio of prey available, all of which influence prey choice, giving a false or incorrect impression of predatory responses in the field. Using molecular methods to study trophic interactions has therefore become increasingly common (King et al. 2008; Sheppard & Harwood 2005; Symondson 2002), as it allows prey consumption to occur naturally in the field, providing direct information about prey choice (Harwood & Obrycki 2013). In liquid feeding species like *A. nemorum* and *A. nemoralis*, molecular screening is the only practical way of analyzing gut-contents, as no identifiable body parts remain. DNA can be extracted from the gut of predators *post mortem*, and analyzed for presence of prey DNA using Polymerase Chain Reaction (PCR). Primers detect a given DNA sequence, amplifying only this fragment of DNA through several PCR cycles, resulting in a high number of DNA copies that can be visually inspected (Erlich et al. 1991). PCR products can be loaded on an agarose gel containing ethidium bromide (or other DNA stains), which will bind to present DNA and be visualized under UV-light. Primers can be designed to be more or less specific depending on study needs, ranging from species specific primers to primers that amplify whole phyla (Agustí et al. 2003b; Chen et al. 2000; Folmer et al. 1994; von Berg et al. 2008; Zaidi et al. 1999). Several successful primers have been developed, and are available for anyone to use.

Several studies show the usefulness of gut-content analysis, and how information obtained can be used to improve biocontrol strategies. Chen et al. (2000) used PCR to identify important cereal aphid predators by amplifying aphid DNA in predator gut-contents, while de Roince et al. (2013) used PCR when investigating whether spiders exerted important early season pest control of aphids in apple orchards. Furthermore, prey detectability half-lives derived from PCR were used to rank the importance of predators on the Colorado potato beetle (*Leptinotarsa decemlineata*) in a study by Greenstone et al. (2007). Other molecular methods used to analyze trophic interactions include the use of monoclonal antibodies (Unruh et al. 2008), next generation sequencing (NGS) (Ekblom & Galindo 2011; Symondson & Harwood 2014) and other PCR based methods, such as quantitative real-time PCR (qPCR) (Weber & Lundgren 2009) and multiplex PCR (Harper et al. 2005; Hatteland et al. 2011)

In this study, the goal was to investigate the field predation of *C. pyri* by *A. nemorum* and *A. nemoralis* in a commercial pear orchard in Norway, in order to gain insight into their potential

as biological control agents. The main objective was to (1) test and optimize previously designed primers for use in a Norwegian study system, in order to study the predator-prey relationship between anthocorid bugs and *C. pyri*. Furthermore, I wanted to (2) verify the role of *A. nemorum* and *A. nemoralis* as important beneficial predators in Norwegian fruit orchards, as no studies (as far as I am aware) have been done to test this in field. Lastly, I wanted to (3) investigate the functional response of *A. nemorum* and *A. nemoralis* to *C. pyri*, by comparing the predation rate (obtained through molecular gut-content analysis) to prey densities. As *A. nemorum* is described as a generalist predator, one would expect predation on *C. pyri* to be mainly density dependent, whereas *A. nemoralis*, as a more specialized predator, is expected to show relatively high predation rates at all prey densities. As such, a difference in pear psyllid predation between *A. nemorum* and *A. nemoralis* collected from the field is predicted.

2. Materials and methods

2.1 Study site

Field samples of *A. nemorum* and *A. nemoralis* were collected from a commercial pear orchard in Lofthus, Hardanger (60°22'0.1"N 6°40'40.5"E) (Figure 5), on the west coast of Norway, from June to mid-August 2015. Lofthus lies along the eastern shoreline of Sørfjorden, the largest fjord arm off the main Hardanger fjord, and is considered one of the most important areas for fruit production in Norway. Mean yearly temperature in Lofthus is 6.8°C, with a mean temperature of 14.3°C during the summer months (June, July and August) (Norwegian Meteorological Institute 2016a). Mean yearly and summer precipitation is 1350mm and 77mm (per month), respectively (Norwegian Meteorological Institute 2016b).

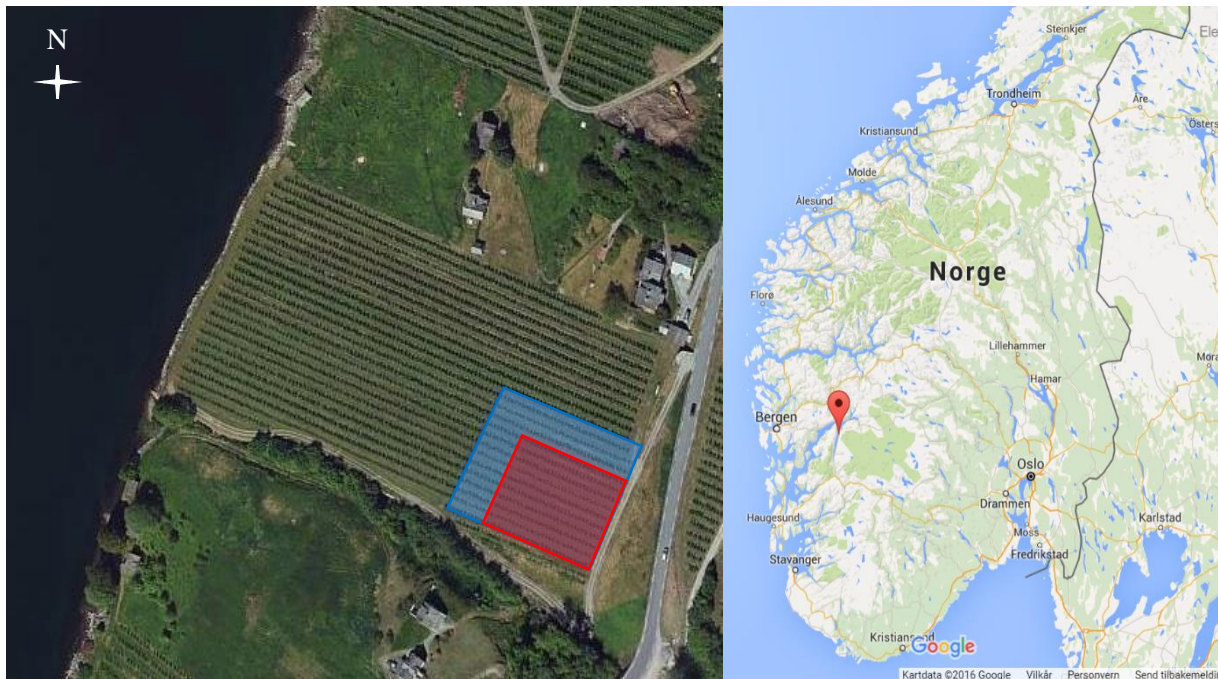


Figure 5: Right: Map of southern Norway with marker indicating location of Lofthus, Hardanger, where field samples were collected (taken from maps.google.com). Left: Aerial photo of the pear orchard/study site, with Sørfjorden to the left in the picture. (taken from www.gulesider.no). The red square indicates the core study site, and the blue outline indicates the buffer zone. The total area of the orchard ~1.8ha, total study site (blue square) ~0.34ha and the core study site (red square) ~0.2ha.

The pear orchard in which this study was conducted is approximately 1.8 ha, with a total of 2500 trees of the cultivar ‘Ingeborg’, planted in 2001 (Figure 5). Surrounding vegetation to the south and east consisted mainly of sparse forest of birch (*Betula* spp.), goat willow (*Salix caprea*) and pine (*Pinus sylvestris*), with stinging nettle and a variety of graminoids.

2.2 Field collection

Insects were sampled from the study site three times during the summer; 5th of June, 1st of July and 13th of August, in order to study the development throughout the season.

The sampling site was divided into 30 plots, with 7 trees per plot and 4 trees between each plot (Figure 6). As pesticides were used in the parts of the orchard not included in the study, a buffer zone was created around the sampling site to prevent contamination (Moreby et al. 1997) (Figure 6). Sampling was done by use of a beating funnel (45x65cm); a randomly selected branch is tapped with a beating stick and all dislodged arthropods are collected in a paper bag at the end of the beating funnel held underneath (Figure 6) (Southwood & Henderson 2000). In each plot, each tree was beaten nine times, three times on three separate branches, for a total of 21 branches per plot. As weather conditions, and especially wet foliage, may affect sampling results, sampling was always performed on sunny, windless days. Sampling started as soon as the sun covered the orchard and morning dew had dried, to ensure that insects were active and to avoid moist foliage. As such, starting time of sampling varied slightly between sampling dates.

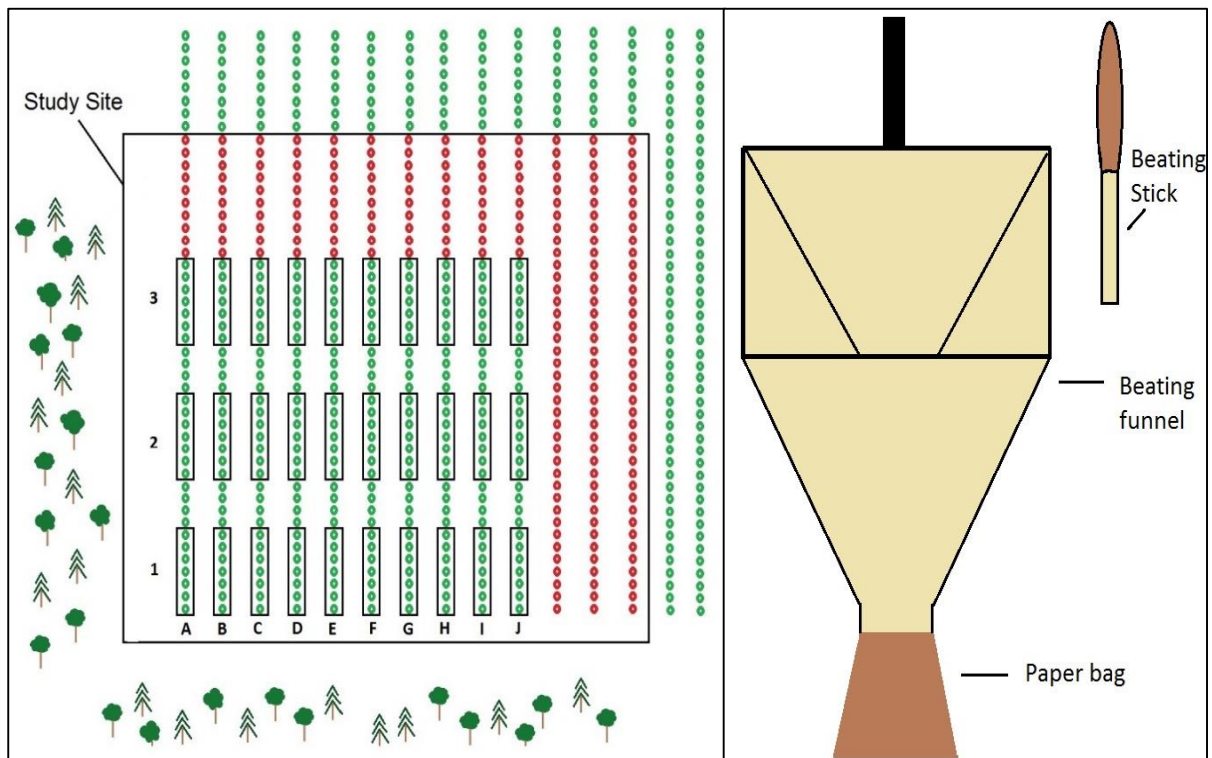


Figure 6: **Left:** Map of the sampling site, showing plot layout and surrounding vegetation. There were 30 plots (black squares) in total, 3 plots on each row of trees, for a total of 10 rows. Each plot consisted of 7 trees (green circles), with 4 trees between each plot. Because pesticides were used in the general orchard surrounding the sampling site, an unsprayed buffer zone was created on either side (marked in red), with 10 trees down and 3 rows across. **Right:** A beating funnel was used to collect samples. A paper bag was attached to the end of the funnel, and a beating stick was used to dislodge arthropods from tree branches into the funnel.

In addition to beating samples, leaf samples were taken in each plot. This was done to avoid underestimation of the *C. pyri* population, as nymphs of *C. pyri* cling to the leaves and as such are difficult to sample with the beating funnel method (pers. comm., B.A. Hatteland, NIBIO 2015). For each plot, 7 leaves were collected from each tree, taking care to sample from low, middle and high hanging branches, for a total of 49 leaves per plot. Leaf samples were always collected on the same day or the day before beating samples.

All samples were immediately put on ice in a cooling box while in the field, to slow digestion of DNA and mitigate false-positives caused by predation in the collection bag (King et al. 2008). Upon return to the laboratory, all samples were frozen and stored at -80°C. Sorting of anthocorids from field samples was performed on ice, as repeated thawing can degrade DNA (sorting 1). All *A. nemorum*, *A. nemoralis* and *Anthocoris* nymphs were placed in separate 1.5mL microcentrifuge tubes and immediately returned to -80°C until molecular assays. *Anthocoris* nymphs were not identified to species, as this is very challenging, and are here on referred to as *Anthocoris*/anthocorid nymphs. Both leaf and beat samples were later sorted through more thoroughly, to identify and count all insects collected (sorting 2).

The number of anthocorids found in the field and the number of anthocorids used in molecular analysis differ for July and August, as several individuals were found in the second sorting that had been overlooked. Because these had been allowed to thaw, they were not suitable for DNA extractions, and were not included in gut-content analysis.

2.3 Feeding experiments

Feeding studies were performed in the laboratory to establish how long after feeding prey DNA is detectable in *A. nemorum*, *A. nemoralis* and *Anthocoris* nymph gut-contents, using the current primers. Feeding studies were conducted for all three anthocorid types to account for any differences in DNA detectability between species and life stages.

A. nemorum and *Anthocoris* nymphs (4-5th instar), as well as *C. pyri*, were sampled during May (*A. nemorum*) and July (nymphs) from nearby pear- and apple orchards not included in this study, to avoid affecting local populations in the study area. Sampling was done by use of beating tray and exhauster. *A. nemorum*/nymphs were kept in plastic boxes, with a damp cotton-ball as water supply and crumpled paper for shelter, in a climate chamber at 15°C with 16:8 hours of light:dark and 70% humidity. The bugs were starved for approximately 60 hours prior to feeding experiments, to prevent false-positives caused by feeding in the field prior to collection. The feeding experiments were conducted in petri dish “arenas” containing damp

filter paper and one *C. pyri*, under the same climatic conditions as the starvation period. Starved bugs were placed in individual arenas and allowed to feed for two hours, during which feeding was monitored by visual inspection every 15 minutes. Afterwards, *A. nemorum* and *Anthocoris* nymphs who had fed were killed by freezing at -80°C , in groups of 10 (of each) at 0, 4, 8, 16, 24, 40 (only nymphs) or 48 hours after feeding. Bugs who had not fed were discarded from the experiment. In addition, 20 unfed, starved bugs (10 *Anthocoris* nymphs and 10 *A. nemorum*) were kept as negative controls. All individuals were placed in separate 1.5mL microcentrifuge tubes and stored at -80°C until molecular assay.

Feeding experiments were also conducted for *A. nemoralis*, using the same protocol as stated above. However, due to unforeseen events, this feeding experiment only included groups of bugs killed at 0, 8 and 48 hours after feeding ($n=10$ for each group except 48h, where $n=9$). *A. nemoralis* used in the feeding experiment were purchased as adults from ©Borregaard Bioplant ApS in Denmark.

2.4 DNA extractions

DNA was isolated in the laboratory from *A. nemorum*, *A. nemoralis* and *Anthocoris* nymphs from both feeding experiments and field collections, as well as from *C. pyri* and non-target organisms used for specificity tests (see below). All insects were identified to species or taxon before DNA isolation. Due to time restrictions, only a subsample of the relatively large number of anthocorid nymphs collected at the last field sampling (18.08.2015) was included in the DNA extractions. The subsamples were made by randomly selecting 20% of the anthocorid nymphs from each plot. All adult bugs were included.

Each individual was placed in a 1.5mL microcentrifuge tube and crushed whole, using micro pestles, in 180 μL ATL buffer (Qiagen) (Zaidi et al. 1999). To avoid contamination between samples, micro pestles were sterilized between each use, using a combination of DNA Away™, chlorine and autoclaving (Champlot et al. 2010). DNA was isolated using Qiagen DNeasy Blood and Tissue Kit, following the manufacturers protocol for animal tissues. During step 2 of the protocol, all samples were incubated on a heat block overnight, to ensure complete lysis. Total DNA was eluted in 100 μL AE buffer, and stored at -20°C until subsequent PCR amplification. To test for possible contamination during extractions, negative controls without DNA were included for all sets of extractions.

2.5 Primers

Two primer sets were used to amplify fragments of the mitochondrial COI (cytochrome oxidase subunit I) gene of psyllids, from the guts of anthocorid bugs (Table 1). Both primer sets were designed by Agustí et al. (2003b) for a study looking at detection of *Cacopsylla pyricola* in predatory gut-contents. The main primer set Cp2F/Cp7R, was designed to amplify *C. pyricola*, but was also shown to amplify *C. pyri*. The other primer set, Cp3F/Cp6R was designed to amplify all species in the Psyllidae family.

As several factors influence primers ability to amplify target DNA, both primer sets were tested on *C. pyri* and *C. pyricola* DNA extractions (same as was used in specificity testing) prior to running field samples, to verify and optimize amplification ability for the current study (Roux 1995; Saiki 1989). The PCR reaction and protocol was based on Agustí et al. (2003b), however, to save time, a premade master mix (Promega GoTaq Green Master Mix) was used as the basis and the PCR reactants were adjusted accordingly.

Table 1: Primers used in this study, designed to amplify fragments of the mitochondrial cytochrome oxidase I (COI) gene in psyllids.

Target species/family	Primer	Primer sequence (5' - 3')	Fragment size (bp)	Reference
<i>Cacopsylla pyricola</i> / <i>C. pyri</i>	Cp2F	CCTTCGTTATATCTTCTCC	271	Agustí et al. (2003b)
	Cp7R	GTAAAAGAAGAAAGGCAGTG		
Psyllidae	Cp3F	GTCTAGTAGACCAAGGAGTAGG	188	Agustí et al. (2003b)
	Cp6R	CTATTGTGTGGAGGTTTCTTC		

2.6 PCR amplification and optimization

PCR amplifications were performed in 12.5µl reaction volumes, containing: 3µl DNA, 6.25µl GoTaq Green Master Mix (Promega) containing 1.5mM MgCl₂, 0.5µl of each primer (10µM), 0.125µl BSA and 1.375µl dH₂O. To increase the concentration of MgCl₂ to 3mM (as was used by Agustí et al. (2003b)), 0.75µl MgCl₂ (25mM) was added, allowing for a higher annealing temperature and specificity (Ampliqon A/S 2016; King et al. 2008). Bovine serum albumin (BSA) was included to optimize the PCR reaction for gut-content analysis, as BSA has been shown to reduce PCR-inhibition in gut-content samples (Juen & Traugott 2006).

Samples were amplified in a BIO-RAD T100™ Thermal Cycler. Cycling conditions were; denaturation at 94°C for 2 min, followed by 35 cycles of 30s denaturation at 94°C, 30s annealing at 58°C and 45s extension at 72°C, and a final cycle of extension at 72°C for 5 min.

The PCR protocol was adapted from Agustí et al. (2003b), and the optimal annealing temperature was verified by running temperature gradient PCRs. The same PCR conditions were applied for both primer sets.

To test for possible contamination during PCR setup, negative controls consisting of PCR reagents with water instead of DNA, were included for each PCR run. In addition, samples with *C. pyri* DNA and DNA of starved anthocorid bugs were included as positive and negative controls, respectively. To minimize contamination, PCR setups were always performed in a dedicated, sterile PCR chamber.

All PCR products were separated by electrophoresis in 1.5% agarose gels (1x TBE buffer) stained with ethidium bromide, run at 90V for 60 minutes and photographed under UV light.

2.7 Primer specificity test

To make sure only target prey DNA was amplified, both primer sets were tested on DNA extractions of other potential prey and non-target organisms, including *A. nemorum* and *A. nemoralis*, found at the study site. All DNA extractions followed the same protocol as previously stated, as did the PCR setup and subsequent gel electrophoresis. In case of larger insects or predators, only part of the insect was used in DNA extractions, taking care to use body parts that did not include gut-contents (e.g. a leg). At least one specimen was tested for each taxon.

2.8 Verification of DNA results

To verify successful DNA extraction and rule out any false negatives due to lack of DNA, all DNA samples were tested using a spectrophotometer (Nanodrop 2000). In addition, PCR was run twice on all negative samples and a random selection (20%) of positive samples, to verify PCR results, as recommended by Sint et al. (2011). In cases where results were inconclusive (e.g. negative on first run and positive on second run), a third PCR run was performed, and final results were based on a “two out of three” principle. In cases where the first PCR run gave such weak bands that the presence of these became subject to personal opinion, a second PCR run was performed, where a positive second run was considered a confirmation of positive result. Samples with repeated weak bands were discarded from the results.

2.9 Statistical analysis

All statistical analysis were performed using R version 3.2.0 (R Core Team 2014) and RStudio version 0.99.893 (RStudio Team 2015). Generalized linear models (GLM) were used to analyze

data from both feeding experiments and fields sampling, and were made using the ‘glm’ function in the ‘stats’ package. As the response variable was either PCR-positive or PCR-negative, a binomial distribution was used. To analyze the effect of time on digestion, a GLM was fitted for each anthocorid type with probability of detection (as the percentage of PCR-positive anthocorids) as the response and time as explanatory variable. The detection half-life (defined as the time when 50% of bugs tested positive for DNA) was calculated using the binomial regression equation ($LD50 = -\text{intercept}/\text{coefficient}$). To test for significant difference in half-life between anthocorid types, a GLM was fitted with PCR-results as response and anthocorid type as explanatory variable.

To analyze the relationship between prey density and predation in the field, GLMs were fitted for each anthocorid type, with the predation (as the percentage of anthocorids positive for psyllid DNA) as the response and number of *C. pyri* nymphs, *C. pyri* adults and *Lasius niger* as explanatory variables. Because beating samples only collected *C. pyri* adults and larger nymphs (4th-5th instar), and leaf samples collected nymphs of all stages (but no adults), beating sample numbers were used for *C. pyri* adults, while leaf sample numbers were used for *C. pyri* nymphs. Because of a high correlation between number of *C. pyri* nymphs and *C. pyri* adults, both variables were not included in the same model. The best model was chosen based on the Akaike information criterion. The ‘allEffects’ command in the ‘effects’ package was used to create effect plots (Fox 2003). To test for differences between *A. nemorum* and *A. nemoralis* predation, a GLM was fitted with predation as response and anthocorid type as explanatory variable.

3. Results

3.1 Primer success and specificity

The primer set Cp2F/Cp7R failed to amplify target prey DNA (*C. pyri*), only producing bands for the closely related *C. pyricola* (Figure 7), for which it was originally designed (Agustí et al. 2003b).

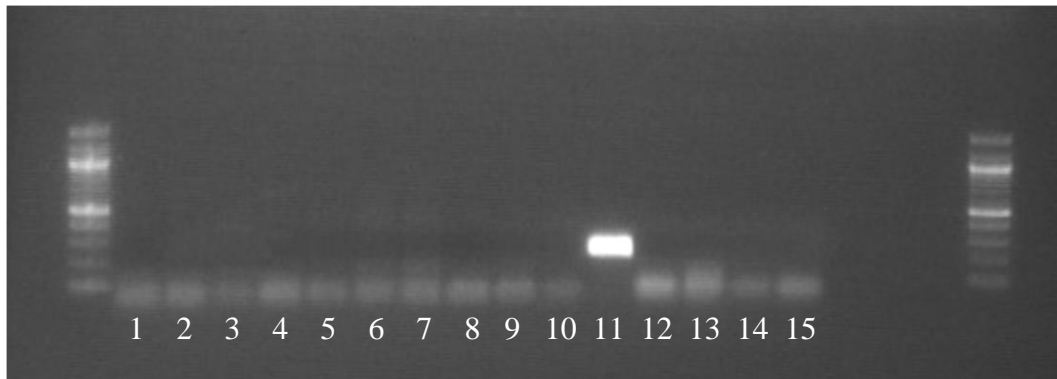


Figure 7: Agarose-gel of PCR amplified DNA using the primer set Cp2F/Cp7R and the PCR reaction as stated by Agustí et al. (2003b). Visible bands are amplified DNA fragments, confirming the presence of target DNA. Lane 1-6: *Cacopsylla pyri* DNA, 7-10: *C. mali* DNA, 11: *C. pyricola* DNA, 12-14: *Anthocoris nemorum* (starved), 15: PCR negative control.

Table 2: Specificity tests were performed on both primer sets (Cp3F/Cp6R and Cp2F/Cp7R), in order to rule out amplification of non-target DNA. The tested taxon included target prey, predators and other potential non-target prey found in the field ($N \geq 1$ for all taxon). Because Cp2F/Cp7R failed to amplify the target prey (*Cacopsylla pyri*), testing of other non-target organisms was not performed for this primer set. - = No amplification, n = not tested.

Higher order/taxon	Family	Species	Cp3F/Cp6R	Cp2F/Cp7R
HOMOPTERA	Psyllidae	<i>Cacopsylla pyri</i>	188 bp	-
		<i>C. pyricola</i>	188 bp	271 bp
		<i>C. mali</i>	188 bp	-
		<i>C. melanoneura</i>	188 bp	-
	Aphididae	<i>Macrosiphum rosae</i>	-	n
AUCHENORRHYNCHA			-	n
HETEROPTERA	Miridae		-	n
	Anthocoridae	<i>Anthocoris nemorum</i>	-	-
		<i>A. nemoralis</i>	-	-
THYSANOPTERA			-	n
COLEOPTERA	Cantharidae		-	n
COLLEMBOLA			-	n
LEPIDOPTERA	Geometridae		-	n
	Tortricidae		-	n
	Noctuidae		-	n
TROMBIDIFORMES	Tetranychidae	<i>Panonychus ulmi</i>	-	n

In addition, none of the other psyllid species tested were amplified with this primer set, indicating species-specific primers. Thus, Cp2F/Cp7R was not used further in the study, and all results are based on the second primer set; Cp3F/Cp6R. This set amplified DNA from all psyllid species tested, including the target prey *C. pyri*, producing the expected bands of 188 bp. Neither predators nor non-target prey were amplified (Table 2).

3.2 Feeding experiments

Psyllid DNA was detected in the gut of *A. nemorum*, *A. nemoralis* and *Anthocoris* nymphs at all post-feeding times. For both *A. nemorum* and *Anthocoris* nymphs, 90-100% of the 10 individuals were positive for prey DNA at every time interval (Table 3) resulting in detection half-lives of 110 and 131.6 hours, respectively (Figure 8a+b). For *A. nemoralis* the detection half-life was 39.6 hours (Figure 8c), given the limited data available. Prey detection time did not significantly differ between the two species ($P = 0.137$, GLM), nor was there a difference between *A. nemorum* and anthocorid nymphs ($P = 0.36$, GLM). However, there was a significant difference in detection times between *A. nemoralis* and anthocorid nymphs ($P = 0.024$, GLM), though as the majority of anthocorid nymphs were likely to be *A. nemorum* it may not be accurate to compare these. Lastly, all negative controls (starved anthocorids, N=20) failed to amplify prey DNA, indicating that the starvation period was adequate.

Table 3: Results of feeding experiments conducted in the laboratory, with percentage of predators positive for psyllid DNA for each post-feeding time. N =10 of each predator for each time group, except *Anthocoris nemoralis* at 48h (N=9), total N = 159. - = no individuals tested for this post-feeding time.

Time post-feeding (hours)	<i>A. nemorum</i> (% positive for prey DNA)	<i>A. nemoralis</i> (% positive for prey DNA)	<i>Anthocoris</i> nymphs (% positive for prey DNA)
0	90	100	90
4	90	90	100
8	90	-	90
16	100	-	100
24	80	-	100
40	-	-	80
48	80	33.3	90

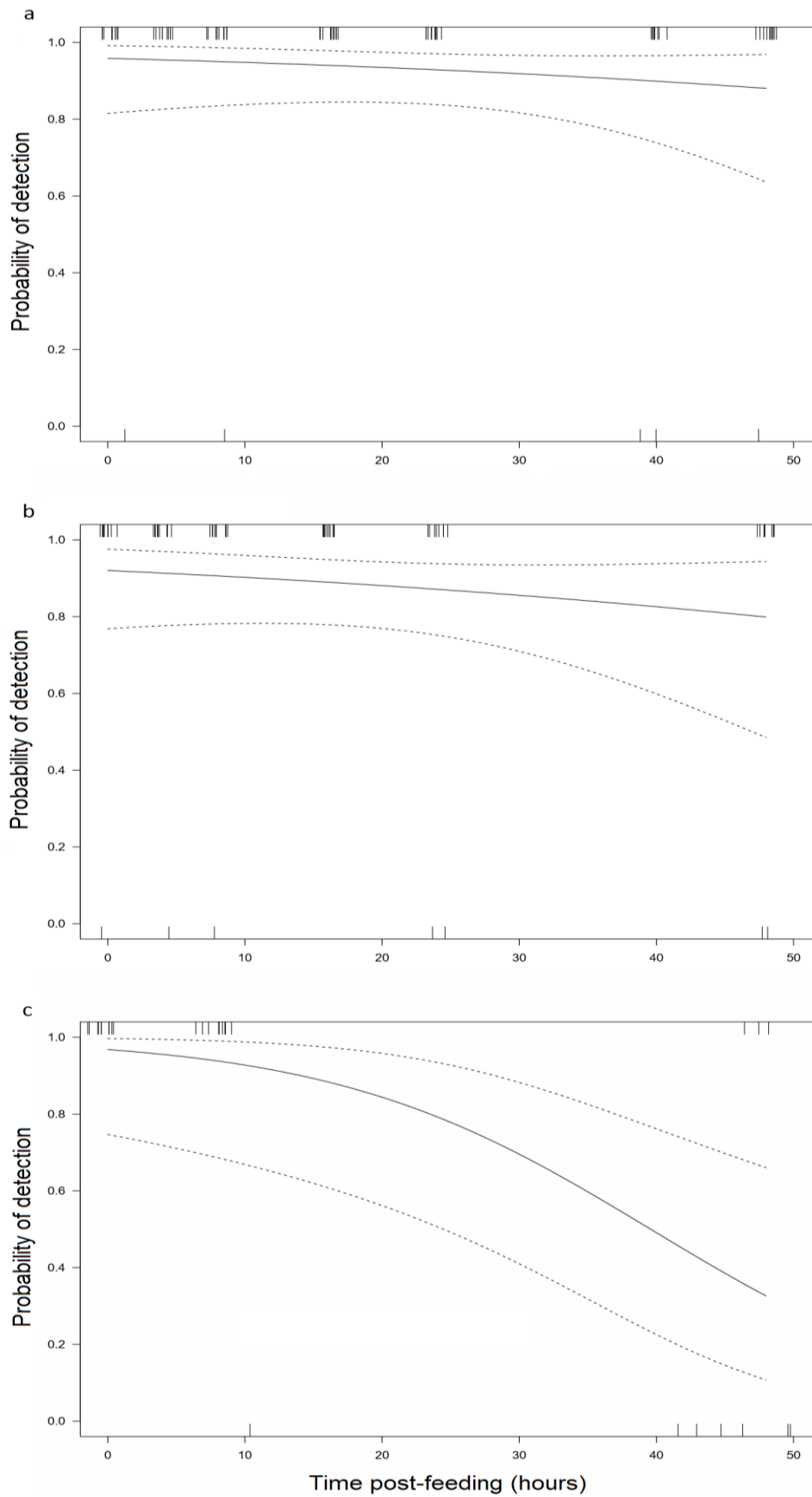


Figure 8: Detection plots showing the relationship between probability of detecting psyllid DNA in predator gut-contents and time since feeding. **a** = Detection plot for anthocorid nymphs (half-life = 131.6 h), **b** = detection plot for *Anthocoris nemorum* (half-life = 110h), **c** = detection plot for *A. nemoralis* (half-life = 39.6 h). The solid lines represent the binomial GLMs, and the dotted lines represent the 95% confidence limits.

3.3 Arthropods found in the field collection

C. pyri was the most numerous species in the field, and constituted 99% of all psyllids found and 89% of all available prey (excluding intraguild predation and not accounting for differences in biomass) (Table 4). *A. nemorum* and *A. nemoralis* were the only anthocorid bugs found in the orchard, and, together with ants, the most numerous predators.

Table 4: Overview of arthropod specimens collected in the field, beating samples and leaf samples pooled. Roughly divided in prey and predator, excluding intraguild predation. Numbers do not account for differences in biomass between individuals/taxon.

Species/Taxon	Total no. of individuals	No. sampled in June	No. sampled in July	No. sampled in August
Prey				
<i>Cacopsylla pyri</i>	3187	280	1414	1493
Psyllid eggs	2211	642	913	656
<i>C. pyricola</i>	2	0	0	2
<i>C. mali</i>	18	1	16	1
<i>C. melanoneura</i>	12	2	3	7
Aphids	302	261	22	19
<i>Panonychus ulmi</i>	2	2	0	0
Thrips (Thysanoptera)	27	24	0	3
Leafhoppers (Auchenorrhyncha)	34	9	1	24
Flower bugs (Miridae)	3	0	0	3
Lepidoptera	7	0	3	4
Predators				
<i>Anthocoris nemorum</i>	280	26	40	214
<i>A. nemoralis</i>	86	7	1	78
<i>Anthocoris</i> nymphs	889	0	172	717
<i>Lasius niger</i> (Formicidae)	1548	478	694	376
<i>Formica fusca</i> (Formicidae)	16	1	11	4
Soldier beetles (Cantharidae)	153	117	23	13
Predatory mites (Acari)	166	0	65	101
Lacewings (Neuroptera)	26	0	0	26
Ladybirds (Coccinellidae)	7	2	1	4
Spiders (Araneae)	148	83	29	36

3. Results

3.4 Field predation

In total, 1255 anthocorid bugs were collected from the field during the season. Molecular gut-content analysis was performed on 585 of these, of which 82% tested positive for psyllid DNA (Table 5). 15 of the tested samples were discarded due to poor DNA extractions, and are not included further in the results.

Table 5: Overview of results from field samples for each sampling time, showing predation (as percentage of anthocorids positive for psyllid DNA in gut-content analysis), mean number of *Cacopsylla pyri* (\pm standard error) and *Lasius niger* in beating funnel samples (*L. niger* not present in leaf samples). T = total number of anthocorids sampled (beating + leaf samples), N = number of anthocorids tested in gut- content analysis (= number of individuals found in first sorting, except for anthocorid nymphs in August that were too numerous to test). S1 = number of anthocorid nymphs in August, first sorting.

Sampling time	Anthocorids	Positive for psyllids (%)	No. of <i>C. pyri</i> nymphs		No. of <i>C. pyri</i> adults		No. of <i>L. niger</i>
			Leaf sample	Beating sample	Leaf Sample	Beating sample	
June	<i>A. nemorum</i> (T = N = 26)	84.6					
	<i>A. nemoralis</i> (T = N = 7)	85.7	3.2 \pm 0.4	4.6 \pm 0.6	0 \pm 0	1.53 \pm 0.2	15.9 \pm 2.1
	<i>Nymphs</i> (T = N = 0)	-					
July	<i>A. nemorum</i> (T = 40, N = 37)	91.9					
	<i>A. nemoralis</i> (T = 1, N = 0)	-	4.7 \pm 0.8	5.1 \pm 0.7	0.4 \pm 0.1	36.9 \pm 2.9	23.1 \pm 5.1
	<i>Nymphs</i> (T = 172, N = 155)	88.9					
August	<i>A. nemorum</i> (T = 214, N = 165)	73.3					
	<i>A. nemoralis</i> (T = 78, N = 68)	82.4	10.4 \pm 1.9	0.2 \pm 0.1	1.7 \pm 0.6	37.5 \pm 4.7	12.5 \pm 1.9
	<i>Nymphs</i> (T=717,S1=544, N=114)	87.7					

Anthocorids positive for psyllid DNA was recorded for all sampling times, with the lowest predation rate (percentage of psyllid-positive anthocorids) being 73.3% (Table 5). Predation rates did not differ between the two *Anthocoris* species, nor between life stages for any of the sampling dates or for the season as a whole. Predation rate by anthocorid nymphs was significantly related to number of *C. pyri* nymphs in August ($P = 0.048$, GLM), with higher predation rates in plots with higher *C. pyri* nymph densities (Figure 9). Predation by *A. nemorum* was also significantly related to *C. pyri* nymph densities in August ($P = 0.023$, GLM) when *L. niger* was included in the model, where *A. nemorum* predation was negatively related to *L. niger* density ($P = 0.029$, GLM)(Figure 10b). To show the functional response of adult *A.*

nemorum to *C. pyri* nymph densities, a plot of the model without *L. niger* as a variable is included (Figure 10a).

Neither anthocorid nymphs nor *A. nemoralis* predation rates were related to *L. niger* densities. Furthermore, there was no significant relationship between *A. nemoralis* predation and *C. pyri* densities for any of the sampling times or for the season as a whole. Lastly, there was no significant relationship between anthocorid predation and *C. pyri* adult density for any of the sampling times.

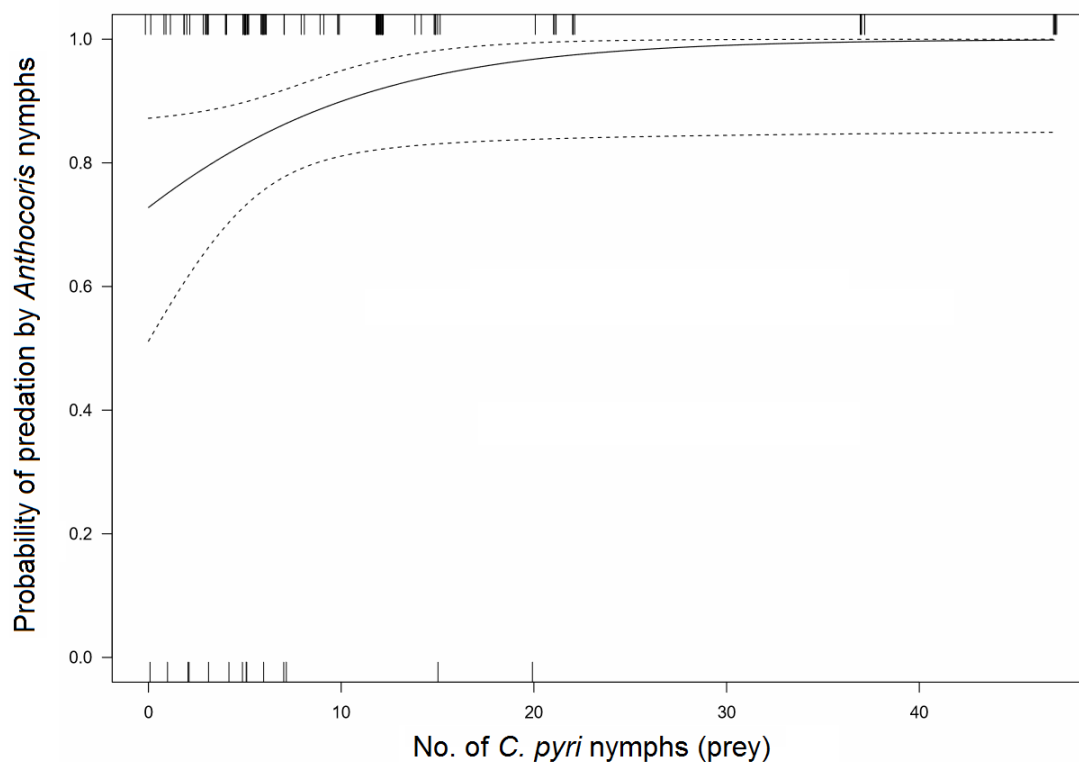


Figure 9: The functional response of *Anthocoris* nymphs to *Cacopsylla pyri* nymphs in the field (in August), with probability of predation given as the percentage of anthocorid nymphs positive for psyllid DNA. Predation by anthocorid nymphs was found to be significantly related to *C. pyri* nymph densities. The solid line represents the binomial GLM, while the dotted lines represent the 95% confidence limits.

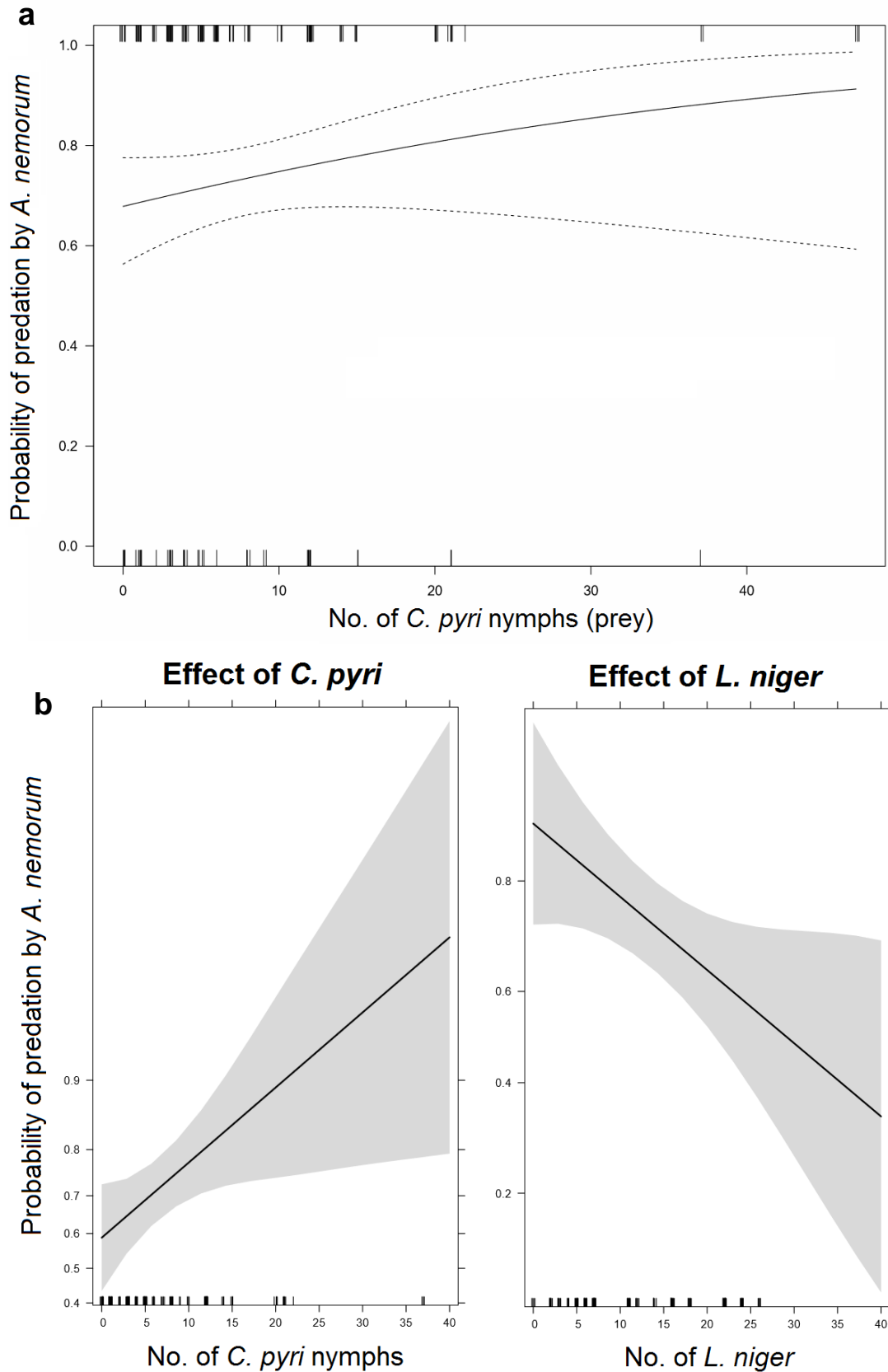


Figure 10: **a:** The functional response of *Anthocoris nemorum* to *Cacopsylla pyri* nymph densities in the field in August, where the probability of predation is given as the percentage of *A. nemorum* positive for psyllid DNA. The solid line represents the binomial GLM, while the dotted lines represent the 95% confidence limits. **b: Left:** The effect of *C. pyri* densities on *A. nemorum* predation, when number of *Lasius niger* are kept constant. **Right:** The effect of *L. niger* densities on *A. nemorum* predation when the number of *C. pyri* nymphs are kept constant. The solid lines show the fitted models, and the grey areas show the 95% confidence limits.

4. Discussion

As far as I am aware, this is the first study applying molecular methods to study field predation on psyllids in European/Norwegian fruit orchards, and so a major part of this study was to test and optimize the molecular method for use in this study system. Though not initially intended, this became the main focus of the study, as many unforeseen obstacles presented themselves during field and laboratory work, and much time was spent optimizing PCR protocols and investigating factors affecting PCR results. This is reflected in the following discussion, emphasizing the PCR method and how field collected data can be interpreted based on molecular half-lives.

The main finding during this method development was that the primer set Cp2F/Cp7R, reported by Agustí et al. (2003b) to amplify DNA from the two closely related psyllid species *C. pyri* and *C. pyricola*, failed to amplify *C. pyri* DNA from the current study system. Further on, the second primer set, Cp3F/Cp6R, did successfully amplify all tested psyllids of the *Cacopsylla* genus, and showed no cross-amplification of other non-target organisms. Using these primers, a surprisingly high predation rate on psyllids by anthocorid bugs was detected for all sampling times, confirming that *A. nemorum* and *A. nemoralis* do predate on psyllids. In addition, the relatively high number of anthocorids present in the field was surprising. *C. pyri* constituted 89% of *Anthocoris* prey available, which indicates that *C. pyri* is an important part of *A. nemorum* and *A. nemoralis* diets.

Contrary to my predictions, there was no significant difference in predation rate (number of anthocorid bugs PCR positive for psyllid DNA) between *A. nemorum* and *A. nemoralis* in the field, indicating that they affect pear psyllid populations in similar ways. Lastly, presence of ants was found to be negatively related to *A. nemorum* predation rates.

4.1 Primer success and specificity

There are several possible reasons why the intended primer set, Cp2F/Cp7R, failed to amplify *C. pyri* DNA in this study, as PCR is a very sensitive method (Agustí et al. 2003b; Chen et al. 2000; Juen & Traugott 2005), and even small alterations can impact results and the presence or absence of bands. To verify that the absence of expected bands was because of failed primers and not errors in the PCR setup or protocol, several factors were adjusted, one at a time. As PCR inhibiting agents may be present in the DNA sample after DNA extraction, dilution of the DNA samples may positively affect PCR results (Juen & Traugott 2006), and is commonly the

first step when troubleshooting PCR (pers. comm., M. Skogen, NIBIO 2015). In addition, the primer concentration in the PCR reaction was adjusted to half the stated amount (Agustí et al. 2003b), as the primers were prone to producing “primer dimers”, possibly indicating that they readily bind to each other rather than DNA at high primer/DNA ratios. Furthermore, the salt concentration in the PCR reaction is crucial, as higher concentrations allow for higher annealing temperatures and thus more specific primers. MgCl₂ was added to the GoTaq Master Mix, doubling the Mg⁺ concentration in the PCR reaction. The latter resulted in a positive result for the *C. pyricola* sample, though the *C. pyri* samples remained negative, indicating that the primers did not amplify the desired prey.

As a last attempt at testing the primer set Cp2F/Cp7R, the PCR reaction was replicated exactly as stated by Agustí et al. (2003b), not using the GoTaq Master Mix. Similarly, this PCR reaction produced a band of the expected fragment size of 271bp, but only for the *C. pyricola* sample (Figure 7). Since the produced band was distinct and clear, it was concluded that the setup was optimal, but that the primers did not amplify *C. pyri* from this population. As there was no recognizable difference between the setup using GoTaq Master Mix with added MgCl₂ and the PCR setup described by Agustí et al. (2003b), the GoTaq Master Mix setup (as stated in the methods) was used when continuing with Cp3F/Cp6R.

One of the more likely reasons why the Cp2F/Cp7R failed to amplify *C. pyri* DNA samples, is due to intraspecific variation in the COI gene the primers were designed to amplify. The COI and COII have been used in several studies (Agustí et al. 2003a; de León et al. 2006; Harper et al. 2005; Harwood et al. 2007; Juen & Traugott 2005; 2006; 2007; McMillan et al. 2007; Sheppard et al. 2004), as these protein encoding genes are less conserved and thus well suited for the design of species-specific primers (King et al. 2008). In addition, as these genes are located in the mitochondrial DNA (mtDNA), several hundred copies of the target gene may be present in each cell (Hoy 2003), increasing primer sensitivity and ability to detect prey DNA in predator gut-contents, compared to primers amplifying nucleus DNA. However, as stated by King et al. (2008), the degree of variation within the target species/group will vary, and should be investigated to identify the most suitable gene region for your target prey group and study. The observed results may indicate a sequence variation in the COI gene between the *C. pyri* population tested in the study by Agustí et al. (2003b) and the population in this study. This is not unlikely, as Agustí et al. (2003b) studied predation on psyllid populations in North America and even small sequence mutations may prevent primers from binding to the target gene.

However, to verify this, *C. pyri* individuals from the current study population would need to be sequenced.

As I was forced to switch to primer set Cp3F/Cp6R, amplifying all species in the Psyllidae family, the inability to distinguish between psyllid species is a potential source of error. However, 99% of all psyllids collected in field samples were morphologically identified as *C. pyri*, and it is assumed that the same will be true for PCR positive anthocorids.

4.2 Feeding experiments

The feeding experiments in this study resulted in much longer detection half-lives than what was found by Agustí et al. (2003b), who reported half-lives of 20.9 and 24.1 hours for Cp2F/Cp7R and Cp3F/Cp6R, respectively (in this study only detection times for Cp3F/Cp6R are reported). This marked difference can be attributed to several factors, as sex (Sunderland et al. 1987; Symondson et al. 1999), life stage, feeding mode (Greenstone et al. 2007; Sunderland et al. 1987), prey and predator species (Chen et al. 2000; Greenstone et al. 2007; Read et al. 2006), fragment length of amplified DNA (Hoogendoorn & Heimpel 2001; Sint et al. 2011; Zaidi et al. 1999) and meal size (Agustí et al. 1999b; Hagler & Naranjo 1997; King et al. 2010) all have been shown to affect DNA detection in gut-content analysis. Several studies have shown the significant effect of temperature on DNA detectability (Hagler & Cohen 1990; Hagler & Naranjo 1997; Hoogendoorn & Heimpel 2001; 2002; Sopp & Sunderland 1989), as temperature affects activity and in turn the digestion rate in predators. Hosseini et al. (2008) performed several feeding studies under different temperatures and found that there was a significant negative effect of temperature on the detection half-life of *Plutella xylostella* (moth) in *Hippodamia variegata* (ladybird) ($P < 0.001$). The same was reported by von Berg et al. (2008), where more than 50% of the carabid beetles (*Pterostichus melanarius*) tested positive for aphid DNA 72 hours post-feeding at 12°C, whereas at 20°C the same was true at 24 h post-feeding. Since the current feeding experiments were conducted at 15°C, to simulate Norwegian field conditions, compared to the 22°C used by Agustí et al. (2003b), at least some of the observed difference in detection half-life is likely attributed to the temperature difference. This suggests the importance of considering temperature when evaluating field collected data, and the usefulness of recording temperature during field sampling.

Several studies also show that detection half-lives vary between different predator species (Chen et al. 2000; Hoogendoorn & Heimpel 2001; Read et al. 2006) and for different prey species in the same predator (Harper et al. 2005; King et al. 2010). When comparing

detectability of the Colorado potato beetle (*L. decemlineata*) in two predators, Greenstone et al. (2007) found detectability half-lives of 7.0 and 50.9 hours for *Coleomegilla maculata* (ladybird) and *Podisus maculiventris* (soldier bug), respectively. Even closely related species have been shown to differ in digestion rates (Symondson & Liddell 1993). This might explain some of the difference between the detectability half-life observed in the current study and that reported by Agustí et al. (2003), since *Anthocoris tomentosus* was used as predator by the latter. However, no significant difference in detection half-lives between *A. nemorum* and *A. nemoralis* was observed in this study

The effect of amplified DNA fragment length on detection half-lives is important to consider. Several studies report a significant positive relationship between detection time and primers amplifying shorter DNA fragments (Agustí et al. 1999a; Chen et al. 2000; Hoogendoorn & Heimpel 2001; Hosseini et al. 2008; von Berg et al. 2008; Zaidi et al. 1999), where DNA detection was more successful when targeting fragments shorter than 300 bp. This is simply because digestion breaks down DNA molecules into smaller pieces, so shorter fragments are likely to stay intact longer, increasing the time primers are able to detect them (King et al. 2008). This corresponds with the shorter detection time reported by Agustí et al. (2003b) for the primer set Cp2F/Cp7R compared to Cp3F/Cp6R, where the former amplified a longer DNA fragment (271bp vs. 188bp).

When designing the feeding experiments in this study, the range of post-feeding times was determined based on the results reported by Agustí et al. (2003b) for Cp2F/Cp7R. Even considering the temperature effect on detection time, a time span of 48 hours was expected to be enough to properly detect the breakdown of psyllid DNA in anthocorid bugs over time. However, since the intended primer set failed to amplify *C. pyri*, the primer set Cp3F/Cp6R was used instead, targeting a shorter DNA fragment. Thus, the predetermined time range proved too short to accurately predict the detection half-life in *A. nemorum* and anthocorid nymphs, as even at 48 hours post-feeding 80 and 90% of bugs were positive for psyllid DNA, respectively. However, by comparing the brightness of bands produced for 24 hours post-feeding to those produced at 48 hours, there is a marked difference, as bands produced at 48 hours are much weaker, indicating lower DNA quantities (Figure 11). Since no distinction is made between “strong” and “weak” bands, but simply “present” or “not present”, this was not taken into account when calculating the detectability half-life.

The reported half-lives for primer set Cp3F/Cp6R are therefore based on the assumption that digestion of prey DNA will continue at the same rate as for the first 48 hours, which is unlikely. Had the feeding experiment covered a longer time range, digestion would likely increase, as these “weak” bands became “not present”, and a better model would produce a more accurate detection half-life. This is supported by the fact that none of the starved bugs (n=20) were positive for psyllid DNA, suggesting a steep downward curve for detection of prey DNA after 48 hours.

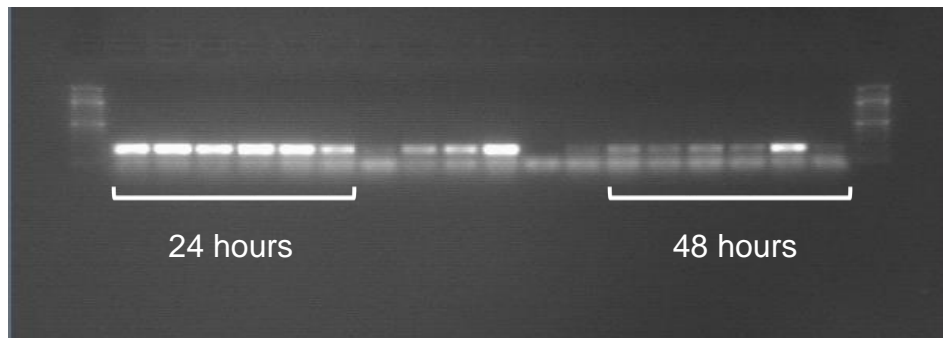


Figure 11: Gel electrophoresis of PCR product from gut-content analysis of *Anthocoris nemorum* used in feeding studies in the lab. Visible bands are amplified DNA fragments, confirming the presence of psyllid DNA. As we don't distinguish between strong and weak bands, this is not taken into account when calculating the detectability half-life (when 50% of bugs are positive for prey DNA), possibly giving a wrong estimation of this. However, it is clear from this gel that bands are weaker after 48 hours of digestion, indicating that there is less DNA in the gut-contents.

Because of the unreliable nature of the observed detection half-lives, assessing the effect of predator species and life stage on detection time is difficult. The detection half-life for *A. nemoralis* is probably the most accurate, as this result is closer to what previous studies report (Agustí et al. 1999a; Agustí et al. 2003a; Greenstone et al. 2007; Sheppard et al. 2004). However, it should be kept in mind that this estimate was based on very few data points. Like Hoogendoorn and Heimpel (2001), no significant effect of life stage on detection time was detected, though new feeding experiments may produce a different outcome. Unruh et al. (2008) found that the probability of detecting pear psyllids in the gut of *A. tomentosus* nymphs was significantly higher than in *A. tomentosus* adults. As *A. nemorum* and *A. nemoralis* are closely related to *A. tomentosus*, and since *Anthocoris* nymphs in this study seem to have a longer (if not significantly so) detection time than adults, this may also be the case here.

Even without accurate detection half-lives, the feeding experiments confirm the successful amplification of psyllids from this study system by the Cp3F/Cp6R primer set, which in itself is an important part of any molecular analysis process, and give an indication of how long prey

DNA is detectable in *A. nemorum* and *A. nemoralis* gut-content under Norwegian conditions. In addition, they highlight how important it is to understand and keep in mind all factors affecting half-lives when evaluating field collected data based on feeding experiments.

4.3 Field predation

Field predation, measured as the percentage of anthocorid bugs positive for psyllid DNA, was found to be high for all sampling dates. Even when recorded densities of *C. pyri* were low, approximately 85% of anthocorids had fed on psyllids. Similar results were found by Unruh et al. (2008), who reported up to 55% of *A. tomentosus* positive for psyllid DNA at psyllid densities below one nymph per leaf. The relatively high percentage of positive anthocorids could be due to the long detection half-lives observed for psyllid DNA in this study. Even if only using the shortest detection time (39.6 hours for *A. nemoralis*), psyllid DNA could still be detectable in the guts of anthocorid bugs for more than three days after the predation event. In addition, it has been shown that meal size and consumption of other prey can increase the detection time of target prey in predator guts (Harper et al. 2005; Symondson & Liddell 1995). Brunner and Burts (1975) found that approximately 8 psyllids per day were needed for *A. nemorum* to attain maturity and produce eggs. Hence, anthocorids in the field are likely to consume more than one psyllid (the amount used in feeding studies) in the detectable time span, increasing the detection time in field data compared to those observed in the lab.

The high percentage of prey positive anthocorids in June and July, when *C. pyri* nymph densities were low, may also be explained by predation on *C. pyri* eggs. The ability of Cp3F/Cp6R to successfully amplify egg remains in anthocorid guts was not tested here, and as such egg numbers have not been included in the density estimation of *C. pyri*, nor in the models predicting predator response to prey densities. However, these results might indicate that psyllid eggs are in fact amplifiable using the current primers, and that *A. nemorum* and *A. nemoralis* predate on psyllid eggs in the field. This is conclusive with results reported by Sigsgaard et al. (2006), where field collected *A. nemorum* and *A. nemoralis* were shown to consume 20.4 and 22.8 *C. pyri* eggs in 24 hours, respectively. Studies have also shown that anthocorid nymphs prefer smaller, immobile prey (Anderson 1962a; Anderson 1962b; Sigsgaard 2010), supporting assumptions of egg predation, at least for early life stages. These results may indicate that *A. nemorum* and *A. nemoralis* exert important psyllid control early in the season. However, few anthocorids were observed in the field in June, in which case even high predation percentages may not have a noticeable impact on *C. pyri* populations. The late arrival of anthocorid bugs in fruit orchards, especially of *A. nemoralis*, has been reported in several studies (Blom et al. 1985;

Scutareanu et al. 1999; Solomon et al. 1989; Souliotis 1999), where pest suppression success is heavily dependent on early migration of sufficient numbers (Solomon et al. 2000). To mitigate pest population growth during the delay before predator arrival, pesticides could be applied in this time window, when negative effects would have minimal impact on natural enemies, as was suggested by Solomon et al. (1989).

Results from the field did not reveal any significant difference in psyllid predation between the two anthocorid species. This is contrary to my predictions and previous studies that describe *A. nemoralis* as a more specialized predator than *A. nemorum* (though both are still considered generalists)(Blom et al. 1985; 2005; Sigsgaard 2010; Solomon et al. 2000). However, determining the percentage of prey positive predators is in itself not a reliable indicator of a predator's relative impact on pest populations (Chen et al. 2000). As described earlier, detection half-lives can be very different for different predators, and should be taken into consideration, especially when comparing two predators as biocontrol agents. For example, if we assume that the detection half-lives acquired in this study were realistic (39.6h for *A. nemoralis*, 110h for *A. nemorum*), then the detection of psyllid consumption would be 2.78 times more likely in *A. nemorum* than *A. nemoralis*. If this is not taken into account, the raw data would in this instance lead us to believe that both predators exert the same per capita impact on pear psyllids, possibly underestimating *A. nemoralis*' predation impact. Chen et al. (2000) suggest giving predators a detectability weighing, where the predator with the lowest detectability would be weighted as 1.0, and all other predators would be given a weight corresponding to the bench-mark half-life divided by that predators own half-life. In this case, *A. nemoralis* would be weighted 1.0, and *A. nemorum* would be 0.36 (39.6h/110h). When comparing *A. nemorum* and *A. nemoralis* in this study, differences in detection half-lives was not adjusted for because of the unrealistic detection results obtained. In addition, *A. nemoralis* and *A. nemorum* are likely to have very similar digestion rate, in which case weighting based on detection half-lives would have little impact on results.

The lack of significant difference between *A. nemorum* and *A. nemoralis* can also be attributed to the fact that the molecular method used here is only semi-quantitative – it gives a proportion of predators that have eaten prey, but does not state the amount of prey consumed by each predator (Zaidi et al. 1999). As *A. nemoralis* is reported to prefer pear psyllids, it may be that prey positive *A. nemoralis* individuals have eaten relatively more psyllids than prey positive *A. nemorum* individuals, in which case *A. nemoralis* predation may be underestimated. However,

as 89% of available prey in the field was *C. pyri*, this was likely the main prey of both predators, allowing for comparisons.

In accordance with the predictions, predation on pear psyllids by *A. nemorum* was significantly related to pear psyllid nymph densities in August. This indicates that *A. nemorum* does not show prey preference towards *C. pyri*, and that increased predation is mainly caused by increased interaction at higher prey densities. Also as predicted, there was no significant relationship between *A. nemoralis* predation and pear psyllid densities at any time during the study. This may indicate that *A. nemoralis* predation on pear psyllids is to a greater extent based on prey preference towards pear psyllids, and that *A. nemoralis* predation on *C. pyri* is relatively high regardless of prey densities. This is supported by the findings of Gundersen (2016), who showed that *A. nemoralis* densities were positively correlated with pear psyllid densities, whereas *A. nemorum* did not show the same response.

Regardless of predator preference, high psyllid predation rates were detected for both anthocorid species, even at low psyllid density, and there was no significant difference in predation rates between the two. This, coupled with the fact that *A. nemorum* arrived in the field earlier than *A. nemoralis*, and was present in much higher densities, suggests that *A. nemorum*, as a generalist predator, is likely as important, if not more so, in regulating *C. pyri* populations in Norwegian pear orchards. This indicates the potential impact of generalist predators on pest populations in fruit orchards, and the importance of considering generalist predators in biological control efforts.

In this study, *A. nemorum* predation on pear psyllids was negatively related to *L. niger* densities, with lower predation rates in plots with more *L. niger* individuals, indicating an antagonistic relationship between *A. nemorum* and *L. niger*. Several studies report of the mutualistic relationship between ants and honeydew-producing Hemiptera (though few have studied this in pear orchards), where ants stimulate the development of large populations, mainly through protection against natural enemies. As a result, ants may show increased aggressiveness, attacking insects that they in the absence of Hemiptera would ignore (Way 1963). Accordingly, Erler (2004) argued that the high number of ants tending *C. pyri* in pear orchards in Turkey decreased the effectiveness of beneficial predators. Hence, the presence of *L. niger* in the current orchard may increase the cost of predating on *C. pyri*, in which case *A. nemorum* may choose to predate on alternative prey. *A. nemoralis* however, as a more specialized predator, might have a higher cost-threshold than *A. nemorum*. This can explain why no relationship between *L. niger* and *A. nemoralis* was observed. Further studies should be done to investigate

the potential impact of *L. niger* on anthocorid predation of psyllids, as it could have a major influence on the effect of *A. nemorum* and *A. nemoralis* as biocontrol agents.

Unfortunately, the molecular method used for analyzing gut-contents of predators in this study does not separate between predation, secondary predation and scavenging. Hence, predation may be overestimated. Because PCR is extremely sensitive to even small amounts of DNA (Juen & Traugott 2005; King et al. 2008; Zaidi et al. 1999), secondary predation can be an important source of error in field data (Hosseini et al. 2008). In a study on detection of secondary predation, Sheppard et al. (2005) found that aphid DNA was detectable in the guts of beetles, who had been fed with spiders who had eaten aphids, for up to 8 hours after consuming the spider. The capacity for intraguild predation in species of the Anthocoridae family has been shown in several studies (Erbilgin et al. 2004; Hill 1957; Meyhöfer 2001; Meyling et al. 2004; Solomon et al. 2000; Tommasini et al. 2002). Still, in the current study system, only a few predators other than anthocorid bugs were observed, and of these only predatory mites are a probable prey for *A. nemorum* and *A. nemoralis* (Heitmans et al. 1986; Solomon et al. 2000). However, studies do report of cannibalism in anthocorid bugs (Hill 1957; Tommasini et al. 2002), especially of younger life stages. Nevertheless, both intraguild predation and cannibalism depend and vary based on several factors such as prey availability, predator densities and predator encounter rates, age distribution and available refugia (Polis et al. 1989; Sheppard et al. 2005). It is therefore difficult to assess the contribution of these factors in the current study system without further studies. This is also the case for scavenging, as anthocorid bugs have been reported of preying on dead individuals (Hill 1957), which was also observed by me in the laboratory. As Juen and Traugott (2005) found that carrion DNA (*Melolontha melolontha*) was just as detectable as fresh prey DNA in the guts of *Poecilus versicolor*, irrespective of carrion age, there is clearly a need to understand and quantify the possible contribution of scavenging to predatory diets in cases where active predation needs to be distinguishable.

Lastly, it is important to note that the summer of 2015 was a very cold and wet summer, with the mean temperature 1.1°C lower than normal (Norwegian Meteorological Institute 2016b), and a mean precipitation of 97.8mm (per month) compared to the normal mean of 77mm (Norwegian Meteorological Institute 2016a). As such, the observed *C. pyri* population was in general noticeably low, to such an extent that pesticides were not needed during the season. As a result, the data collected here do not reflect a pear orchard system with an actual pest problem, and consequently may not properly show predator reactions to a pest outbreak. However, the

results clearly support the assumption that *A. nemorum* and *A. nemoralis* are important beneficial insects in Norwegian fruit orchards, and that they both predate on pear psyllids and most likely exert important pest control.

4.5 Future focus

Molecular methods like standard PCR, multiplex PCR and Next Generation Sequencing (NGS) make it possible to study and unravel trophic interactions that before were impossible or next to impossible to observe in the field. However, the importance of understanding how different factors, both abiotic and biotic, affect detection times and as a consequence how we interpret field collected data, are clearly shown in this discussion. There are many aspects of the predator-prey system studied here that need to be explored further to better understand their trophic interaction in the field, how this affects interpretation of field data and what it means for potential biological control.

Future focus should be on designing primers that amplify *C. pyri* DNA from Norwegian/European populations, and determining the detection half-lives of *C. pyri* in *A. nemorum* and *A. nemoralis* gut-contents. This will provide more accurate estimates of the detection time than those obtained in this study, and field data can be corrected for potential differences between predator species, resulting in a better assessment of predator impact. In addition, the effect of temperature should be explored, as temperature not only affects digestion rate, but may also cause behavioral changes. Simonsen et al. (2009) showed that predation by *A. nemorum* on cabbage aphids (*Brevicoryne brassicae*) increased at higher temperatures, from 6.9 to 15.9 aphids per day at 12°C and 20°C, respectively. Since temperature fluctuates greatly in Norway, as was seen during this study where recorded minimum and maximum temperatures during the summer were 4.4°C and 28.3°C (Norwegian Meteorological Institute 2016b), respectively, temperature is likely an important factor to consider when analyzing field results collected in this area. Furthermore, the effect of sex and life stage (nymphs vs. adults) should be determined, as behavioral differences in food intake or activity can affect digestion rates (Harwood et al. 2009). However, there would be a problem of conveying this in laboratory experiments, as bugs are kept in a restricted arena during testing, and as such would not show the same behavior as in the field.

Since *A. nemorum* is considered a more generalist predator than *A. nemoralis*, *A. nemorum* individuals are likely to consume a larger variety of species. As predatory impact on prey populations depend on the amount of prey consumed, determining prey composition and the

relative amount of target pest consumed by each predator species, is essential when assessing the effectiveness of a predator as a biocontrol agent. By using either multiplex PCR or NGS, the range of prey consumed by an individual predator can be determined (Harper et al. 2005; Hatteland et al. 2011; Symondson & Harwood 2014). In NGS, the gut-contents of an anthocorid bug can be analyzed, resulting in a collection of gene sequences for all prey species present in the sample (Ekblom & Galindo 2011). In contrast to PCR based methods, NGS does not require previous genome information on potential prey species. If genome information is available, multiplex PCR is a great alternative. When using multiplex PCR, several primers amplifying different prey DNA (resulting in different sized bands on agarose gel) are run simultaneously, allowing for more information to be obtained per PCR run, and thus more ecological insight into predator diets. Either method would provide key information on *A. nemorum* and *A. nemoralis* diets, potential differences between them, and the relative importance of *C. pyri* as a food source for each predator.

As stated in the discussion, psyllid eggs may be an important food source for anthocorid bugs, especially early in the season. Anthocorid predation on psyllid eggs should therefore be investigated, since high consumption rates may delay or prevent pest outbreaks. In addition, future studies should explore the arrival time of *A. nemorum* and *A. nemoralis* in pear orchards, from where and how they are attracted to orchards and potential IPM strategies to expedite their arrival, as biocontrol success is heavily dependent on early arrival of sufficient numbers of predators.

Lastly, as species identification of anthocorid nymphs is difficult upon visual inspection, molecular methods like multiplex PCR or NGS can be used to identify anthocorid individuals by analyzing DNA samples. This would save time, as individuals would not have to be identified prior to DNA extractions, and would allow for better study of differences between life stages, as anthocorid nymphs can be identified to species.

5. Conclusion

The primer set Cp2F/Cp7R was unable to amplify *C. pyri* DNA from the current study system, most likely because of genetic differences between Norwegian populations and populations in USA tested by Agustí et al. (2003b). New primers should be developed for future studies targeting Norwegian (and probably European) populations. In addition, I have shown the importance of performing feeding studies, and how factors affecting detection half-lives should be investigated and kept in mind when interpreting field data.

The high predation rates for anthocorid bugs observed in this study, even at low *C. pyri* densities, indicates that they are important beneficial predators in Norwegian pear orchards, and that both *A. nemorum* and *A. nemoralis* contribute to *C. pyri* control. Furthermore, *A. nemorum* arrived earlier, was present in higher densities and showed equally high predation rates in the field as *A. nemoralis*, suggesting that *A. nemorum* as a generalist predator may be as important, if not more so, in regulating *C. pyri* populations in Norwegian orchards.

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