Experimental methods to support the risk assessment of invertebrate biological control agents

by

ir. Sara Maes

Thesis submitted in the fulfillment of the requirements for the Degree of Doctor (PhD) in Applied Biological Sciences
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BCA</td>
<td>biological control agent</td>
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<td>Df</td>
<td>degrees of freedom</td>
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<td>ERA</td>
<td>environmental risk assessment</td>
</tr>
<tr>
<td>ERBIC</td>
<td>Evaluating Environmental Risks of Biological Control Introductions into Europe, a project funded by the European Commission</td>
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<tr>
<td>IAS</td>
<td>invasive alien species</td>
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<td>IBCA</td>
<td>invertebrate biological control agent</td>
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<tr>
<td>L:D</td>
<td>light-dark cycle expressed in hours (e.g. 16:8 indicating 16h photophase and 8h scotophase)</td>
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<td>N</td>
<td>number of sampled individuals</td>
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<td>P</td>
<td>significance of statistical test</td>
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<tr>
<td>R</td>
<td>Pearson’s correlation coefficient</td>
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<td>REBECA</td>
<td>Regulation of Biological Control Agents, a project funded by the European Commission</td>
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<td>RH</td>
<td>relative humidity</td>
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<td>SE</td>
<td>standard error</td>
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<td>SPSS</td>
<td>Statistical Product and Service Solution</td>
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Chapter 1

Introduction, objectives and thesis outline


1.1 Introduction

Invasive alien species (IAS), alongside climate change, habitat destruction, pollution and overexploitation, are considered one of the main causes of global diversity loss. IAS coupled with climate change have even been referred to as a ‘deadly duo’ (Roy et al. 2011). The number of alien species in Europe was recently documented by the DAISIE-project (Delivering Alien Invasive Species Inventories for Europe, http://www.europe-aliens.org). According to the data available in the DAISIE database, 1341 arthropod species were introduced unintentionally, whereas the introduction of 218 arthropod species was intentional. Almost all intentionally introduced species were released in a programme of biological control of insect pests or weeds (Rabitsch 2010; Engelkes and Mills 2011).

The vast majority of exotic (non-native) biological control agents (BCAs) do not present ecological or societal problems and have been proven to be beneficial to the economy (De Clercq et al. 2011). However, discussion about potential side-effects of the release of exotic natural enemies flared up in the 1990s after the UN Convention on Biological Diversity (CBD) was drafted and the first serious cases of non-target effects were reported (Follett and Duan 2000; Wajnberg et al. 2000; van Lenteren 2012). As it is generally accepted that once a released species gets established it is extremely difficult to mitigate or eradicate, the CBD advocates to strictly prevent or control the introduction of non-indigenous species (Messing et al. 2006; Kenis et al. 2008; Roy et al. 2011).

As a result, there is a tendency in biological control nowadays to first look for indigenous natural enemies when a new exotic pest establishes and to replace exotic BCAs if possible by indigenous species (van Lenteren 2012). Nevertheless, exotic invertebrates are still used in biological control for several reasons (De Clercq et al. 2011). First of all, an exotic natural enemy might be necessary to obtain an effective suppression of an exotic pest. Second, exotic natural enemies may be more successful under the climatic conditions used in protected cultivation than native species. Furthermore, the successful use of certain natural enemies in other parts of the world may incite
biological control producers to use them for local problems (De Clercq 2002; van Lenteren et al. 2003; De Clercq et al. 2011).

The development of a scientifically based methodology for conducting environmental risk assessments (ERAs) is one of the cornerstones to constrain the risks associated with the import and release of exotic BCAs. Two European initiatives, ERBIC (Evaluating Environmental Risks of Biological Control Introductions into Europe) and REBECA (Regulation of Biological Control Agents), have formulated general guidelines to perform ERA testing, integrating information on a candidate BCA’s potential to establish (overwinter), its abilities to disperse, its host range, and its direct and indirect effects on non-target organisms (Figure 1)(van Lenteren et al. 2003, 2006a; Ehlers 2011).

![Flowchart for an ERA of an IBCA](after van Lenteren et al. 2008).

**Figure 1:** Flowchart for an ERA of an IBCA (R: release recommended, NR: no release recommended).
For the assessment of an insect’s overwintering capacity a standard protocol has been developed. Several laboratory procedures were designed to investigate the cold tolerance of an exotic natural enemy, and the relationship between the measured parameters and the chances of survival in the field during winter appears sufficiently robust for an assessment to be based on laboratory studies alone (Hatherly et al. 2005; Boivin et al. 2006; Bale 2011a). In contrast, the general framework for risk assessment elaborated by ERBIC and REBECA has hardly been translated into concrete experimental methods for the study of a candidate BCA’s host range spectrum and its capacity to disperse (Babendreier et al. 2005; Mills et al. 2006; Brown et al. 2011). The MACROREG project funded by the Federal Belgian authorities had as a main goal to support the development of an experimental methodology for the different aspects of a risk assessment for IBCAs, including its establishment potential, host range and dispersal capacity, and to identify potential factors that may affect the outcome of an ERA testing procedure.

Both biotic and abiotic factors may change the fitness of a natural enemy and may thus complicate the evaluation of its safety in the framework of an ERA. For instance, in the laboratory insects are usually cultured under continuous summer conditions (23-27°C, long days). In the field, however, insects may become more cold tolerant in response to environmental cues such as decreasing temperature or photoperiod (Block 1990; Danks 2007). Exposing insects taken directly from laboratory cultures to low temperatures may thus lead to inaccurate predictions of their cold tolerance. Further, natural and laboratory produced populations of a natural enemy may differ in their associations with micro-organisms, including endosymbiotic bacteria. As bacteria have been reported to play a role as ice nucleating agents in insects (Lee et al. 1991; Worland and Block 1999), the cold tolerance of insects with a different bacterial load might differ. Finally, foods used in commercial insectaries may have a strong impact on the physiological responses of the natural enemies produced. Unnatural (factitious or artificial) foods may change the fitness of a natural enemy (Grenier and De Clercq 2003) and may thus also influence its responses to climatic challenges and its dispersal capacity.
1.2 Objectives and thesis outline

The overall objective of this study was to evaluate the potential impact of (a) biotic factors related to the rearing of a natural enemy on different aspects of its ERA using a case-study approach. Two economically important natural enemies, the predatory bug *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) and the mealybug destroyer *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae), were selected as case studies. Specific research goals were:

- inventorying the invertebrate BCAs commercially available to European growers and estimating the proportion of exotic species out of the total assortment
- determining the influence of climatic conditions and diet on the cold tolerance of *M. pygmaeus* and *C. montrouzieri*. Whereas *M. pygmaeus* can be easily reared on both factitious foods and artificial diets (Vandekerkhove et al. 2006), the introduction of alternative diets in the mass production of *C. montrouzieri* was complicated by the lack of a suitable oviposition substrate. Therefore, the development of an alternative rearing method was of crucial importance to achieve the overall objective of our study. Because the infection status of *M. pygmaeus* with endosymbiotic bacteria is well studied and techniques for curing are available (Machtelinckx et al. 2009, 2012), this case study created a unique opportunity to test the effects of endosymbionts on cold tolerance.
- evaluating a computer-monitored flight mill as a tool for assessing the dispersal potential of coccinellid predators and determining the effect of diet on the flight capacity as measured in the flight mill
- studying the prey range of *C. montrouzieri* by applying the general guidelines elaborated by ERBIC and REBECA for host range testing.

Chapter 2 provides an overview of the literature on ERA, *C. montrouzieri* and *M. pygmaeus*. In Chapter 3 the products of European producers of natural enemies were inventoried and the original distribution of the species listed was investigated. An alternative, semi-artificial rearing method for *C.
Chapter 1

C. montrouzieri is presented in Chapter 4. The following four chapters report on the different aspects of an ERA testing procedure (Figure 1). Chapter 5 focuses on the cold tolerance of C. montrouzieri and the effect of diet and climate on its overwintering potential. The usefulness of the flight mill apparatus as a tool in risk assessment using several predatory ladybirds as case studies was assessed in Chapter 6, as well as the influence of diet on the outcome of the experiments. Here, the performance of naturally vs. artificially reared C. montrouzieri was compared to that of the native, two-spotted ladybird Adalia bipunctata (L.) and the invasive, harlequin ladybird Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae). In Chapter 7 the prey range of C. montrouzieri was studied using laboratory feeding experiments. The influence of acclimation, endosymbionts and diet on the overwintering potential of M. pygmaeus was laid out in Chapter 8. The study concludes with a general discussion and some further research perspectives in Chapter 9.
Chapter 2

A literature review
2.1 Exotic biological control agents

2.1.1 Definitions

An exotic species can be defined as a species found in a given area due to (accidental or intentional) introduction and that might survive and subsequently reproduce (Richardson et al. 2000). Throughout this manuscript we align to this definition and use ‘alien’, ‘non-native’, ‘non-indigenous’ and ‘foreign’ as synonyms for exotic.

Because national borders are clearly defined, exotic species of BCAs are often considered to be those not originating from the country where release is intended. However, it is well known that an exotic species, once established, will spread to the limits of its ecological tolerance. Therefore, questions can be raised about the legitimacy of considering countries as an ecologically relevant entity when considering the risks of releasing a BCA. The ecoregion concept is believed to be more relevant here, but different definitions and classifications do appear in literature (Cock et al. 2006; De Clercq et al. 2011). A generally accepted definition for ecoregion is an area of similar climate, landform, soil, potential natural vegetation, hydrology, or other ecologically relevant variables (US-EPA 1996; Clark et al. 1998; Cock et al. 2006). Usually, the first parameter considered in an ecoregion classification is climate, particularly patterns of temperature and rainfall. Bailey (1996) defined the four ecoclimatic zones of the earth, which classifies Europe to the Urals as ‘humid temperate’. When modifying this system by important factors such as altitude, soil and vegetation system, more ecoregions can be defined (Cock et al. 2006). The most recent classification of ecoregions in Europe ‘The Biogeographical Regions of Europe’ is based on vegetation mapping (EEA 2011)(Figure 2). One ecoregion can cover several countries, whereas other countries are divided into two (Belgium, Portugal) or even 3 ecoregions (Spain, Italy, Norway). This approach was used in the Habitats Directive 92/43/EEC and for the EMERALD Network set up under the Convention on the Conservation of European Wildlife and Natural Habitats.
Further, in the framework of the new European regulation 1107/2009/EC concerning plant protection products, Europe has been divided into 3 zones based on agricultural, plant health and environmental (including climatic) conditions. Although the intention was to follow an ecoregion approach, the latter classification is still largely built on national borders. For example, Zone A (North) consists of Denmark, Estonia, Latvia, Lithuania, Finland and Sweden. Following member states belong to Zone B (Centre): Belgium, Czech Republic, Germany, Ireland, Luxembourg, Hungary, Netherlands, Austria, Poland, Romania, Slovenia, Slovakia and United Kingdom. Finally, Zone C (South) consists of Bulgaria, Greece, Spain, France, Italy, Cyprus, Malta and Portugal (EU 2009).

Figure 2: Biogeographical Regions in Europe (source: www.eea.europa.eu).
2.1.2 Benefits of exotic biocontrol agents

2.1.2.1 Use in classical biological control

Classical biological control involves the introduction of small numbers of a natural enemy to reach an equilibrium between pest and BCA at a level below the economic threshold (Bale et al. 2008). The procurement of exotic natural enemies to suppress pest populations has long been an integral part of the biological control of exotic invaders, which often did not encounter effective natural enemies outside their native range. Therefore, co-evolved natural enemies were sought in the pest’s region of origin and in areas that include a climate similar to that of the destination (Van Driesche and Bellows 1996).

In the past, numerous successes of biological control have been reported, also in cases when pesticides proved to be inadequate (Van Driesche and Bellows 1996; Bellows and Fisher 1999). A classical example of the latter is the successful control of the cottony cushion scale *Icerya purchasi* Maskell (Hemiptera: Margarodidae) at the end of the 19th century in California, USA. This scale was unintentionally imported from southern Australia on *Acacia* plants and became resistant to hydrogen cyanide treatments. Two natural enemies were collected in the scale’s native range: the vedalia beetle *Rodolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae) and the parasitoid fly *Cryptochaetum iceryae* (Williston) (Diptera: Cryptochaetidae). Both *R. cardinalis* and *C. iceryae* were released in small numbers in Californian citrus groves and achieved control of the scale in less than 2 years, the ladybird being the most effective agent (DeBach 1964; Bale et al. 2008). Other important successes were the introduction of the herbivorous scale *Dactylopius ceylonicus* (Green) (Hemiptera: Dactylopiidae) against the cactus *Opuntia vulgaris* Miller (Cactaceae) in southern India, and the release of the parasitoid *Apoanagyrus lopezi* De Santis (Hymenoptera: Encyrtidae) for control of the cassava mealybug *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae) in Africa (Neuenschwander 1996; Van Driesche and Bellows 1996). These successes encouraged the use of
exotic natural enemies as a cost effective strategy for the control of exotic weeds and arthropod pests.

The introduction of exotic agents in classical biological control was historically viewed as an environmentally benign approach yielding huge economic and ecological benefits (van Lenteren et al. 2006a; Moeed et al. 2006; Bale 2011a; De Clercq et al. 2011). The benefit to cost ratio of the control of the cassava mealybug *P. manihoti* in Africa was estimated to be in the range of 200:1 to 740:1, depending on the market price of cassava (Zeddies et al. 2001). Although the average cost-benefit ratio of all importation programs is undoubtedly lower than the one calculated for *P. manihoti*, the overall benefits from successful projects outweigh the combined costs of unsuccessful projects. Besides, the economic benefits of successful classical biological control continue to accrue annually (Orr 2009; Hoddle and Syrett 2002; De Clercq et al. 2011). In contrast to chemical control, biological control can be permanent as natural enemies are self-propagating. Another overriding benefit of classical biological control is that natural enemies and targets are co-evolving continuously, making the development of resistance virtually impossible (van Lenteren and Woets 1988; Bale et al. 2008; De Clercq et al. 2011). Moreover, this pest management approach has led to the protection of biodiversity and has aided in restoring natural systems and preserved ecosystems affected by adventive species. For example, the invasion of purple loosestrife, *Lythrum salicaria* L. (Lythraceae), into North American freshwater wetlands has altered nutrient cycling, which led to reductions in plant diversity and reduced habitat suitability for several specialized wetland bird species. The introduction of host-specific weed BCAs has resulted in dramatic declines of purple loosestrife and the once monotypic stands of *L. salicaria* are replaced by a diverse wetland plant community (Blossey et al. 2001).
2.1.2.2 Use in augmentative biological control

Augmentation refers to the release of mass produced natural enemies once or several times per season. These natural enemies may be indigenous or exotic to the area of release (Bale et al. 2008; van Lenteren 2012). In many cases, the use of non-native beneficials is required to control exotic pests. Several pests that now occur under protected cultivation in temperate areas were originally introduced from (sub)tropical climates and may require co-evolved natural enemies for successful biological control. Also, exotic natural enemies may be preferred as they are more effective under the climatic conditions prevailing in protected cultivation than native natural enemies. Furthermore, the cost of production and distribution of a natural enemy may be an important consideration. In some cases, globalization of the marketplace has sparked the demand for exotic species. This demand may be inspired by reports of successful control in other parts of the world, but may also be catalyzed by the mere fact that a beneficial organism is commercially available elsewhere (where it may be indigenous) and is believed to have potential for the local market (De Clercq 2002; De Clercq et al. 2011).

The first success of augmentative biological control in protected cultivation involved the glasshouse whitefly *T. vaporariorum* and the parasitic wasp *E. formosa*. Both species are believed to originate from (sub)tropical America and were accidentally introduced into North America and Europe (Bale et al. 2008). The phytoseiid *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) is another economically important natural enemy that has been used since the late 1960s to manage the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) in a variety of crops. This predatory mite is thought to have a naturally Mediterranean distribution, but commercial stocks might originate from a Chilean population that was accidently introduced into Germany in the late 1950s (McMurtry et al. 1978; Griffiths 1999; De Clercq et al. 2011). For both *E. formosa* and *P. persimilis*, sophisticated application schemes have now been developed, involving the commercial mass production of these BCAs, flexible release plans (frequency and density), introduction of the
pest species to maintain natural enemy populations when required, and manipulation of the climatic
environment (temperature and humidity) to optimize control (van Lenteren 2000).

Since far fewer insecticides can be used under practices of pollination with bumble bees, the release
of natural enemies is a valuable alternative for chemical pest control in crops depending on biological
pollinators. Effective biological control programs suppressing economically important target
organisms have yielded direct monetary benefits to growers. Additional benefits have resulted from
reduced pesticide usage, lowering acute and chronic impacts of chemical pesticides on humans and
the environment. Exotic BCAs have also played a central role in the management of key pests with a
propensity to develop resistance against insecticides, like spider mites, thrips, aphids and whiteflies
(Bale et al. 2008; van Lenteren 2008; De Clercq et al. 2011).

2.1.3 Risks of exotic biocontrol agents

Potential risks associated with the introduction of exotic BCA can be considered in three categories:
human and animal health, plant and crop damage and the environment, of which the potential risks
to the environment are generally believed to be the most important (van Lenteren et al. 2003,
2006a; De Clercq and Bale 2011).

2.1.3.1 Human and animal health

Personnel involved in the production of natural enemies are most likely to be exposed and
protection measures can be introduced to minimise such risks. However, there have been very few
reports of allergies in personnel working in the biocontrol industry, and any species that caused
problems would be rapidly withdrawn (De Clercq and Bale 2011). Although the health risk of IBCAs
for humans and animals is generally considered to be very low (van Lenteren et al. 2006a), the
invasive harlequin ladybird *H. axyridis* received some attention regarding its potential impact on
human health. This coccinellid has been reported to invade houses, causing problems when reflex
blooding adults stain furnishings and walls (Figure 3). Exposure to reflex blood and occasional bites
may in a small number of cases trigger allergic reactions in sensitive persons, including
rhinoconjunctivitis, asthma, urticaria and angiodema (Kovach 2004; Koch and Galvan 2008; Goetz
2009).

![Aggregation of adult H. axyridis ladybirds during their overwintering in houses (source: www.pestic.msu.edu).](image)

**Figure 3:** Aggregation of adult *H. axyridis* ladybirds during their overwintering in houses (source: www.pestic.msu.edu).

2.1.3.2 **Plant and crop damage**

Non-native natural enemies may affect the health of both cultivated and wild plants in a direct or
indirect manner (Albajes et al. 2006). Introduced weed BCAs may become ineffective if heavily
attacked by released natural enemies, which might result in reduced weed suppression and
consequent damage in natural ecosystems (Goeden and Louda 1976; van Lenteren et al. 2006a). A
dominant predator like *H. axyridis* may also have an indirect impact on plant health. Predation
among individuals living in the same trophic level (intraguild predation) may result in a reduction in
predation pressure on the target pest population and an overall reduction in biological control
(Rosenheim et al. 1995).

Facultatively phytophagous natural enemies may cause direct crop damage. Some mirid predators,
e.g. *M. pygmaeus*, only cause plant damage under very specific circumstances such as high predator
densities combined with low prey availability (Malausa and Trottin-Caudal 1996; Van Schelt et al. 1996; Sampson and Jacobson 1999). *Nesiocoris tenuis* Reuter (Hemiptera: Miridae), generally regarded as a beneficial predator in Sicily and Spain, has been mentioned as a pest in Egypt and in the south of France (Castañé et al. 2011). Feeding by this predator on tomato causes necrotic rings on stems, petioles and flower stalks and might lead to a reduction in vegetative growth and fruit yield (Sanchez et al. 2006; Sanchez and Lacasa 2008; Calvo et al. 2009; De Puysseleyr 2014). *Harmonia axyridis* has been reported to feed in autumn on grapes, apples, peaches, plums, pears, pumpkins and raspberries. However, this frugivory in most cases primarily seems to occur on previously damaged fruits. The wine industry in North America experiences damage as the beetles complicate the wine harvest and production by getting entangled in grape clusters, which are then harvested and processed (Galvan et al. 2008; Koch and Galvan 2008); there are no such reports in Europe (De Clercq et al. 2011).

2.1.3.3 Environment

The potential environmental risks associated with the introduction of exotic BCAs are listed in Table 1 (van Lenteren et al. 2006a). Until now, there is no consensus on how to judge the magnitude of non-target effects and whether these effects can be tolerated or are unacceptable. First approaches have been outlined by Lynch et al. (2001) who suggested a severity index ranging from 0 (no negative reports) to 9 (large scale extinctions of non-target organisms). van Lenteren et al. (2006b) suggested that occasional feeding on a non-target species does not have to affect the abundance of the non-target, and therefore, should not be regarded a problem. Besides, even when a BCA somehow affects the distribution of a non-target organism it is unlikely that it will lead to the worst case scenario: (local) extinction. They argued that all other possible impacts are relatively mild and may be reversible. van Lenteren et al. (2008) further recommended to distinguish between native and exotic
non-targets: if a BCA affects exotics (other than those introduced on purpose), the impact should not be considered negative.

<table>
<thead>
<tr>
<th>Potential risk</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extinction of (non-)target species</td>
<td>Van Nouhuys and Hanski 2000; Jung and Croft 2001; Nathan et al. 2003</td>
</tr>
<tr>
<td>Reduction in population levels of native species</td>
<td>Jung and Croft 2001; Nathan et al. 2003; Aubert and Quilici 1983</td>
</tr>
<tr>
<td>Transmission of pathogens harmful to native organisms</td>
<td>Hart et al. 1997</td>
</tr>
<tr>
<td>Competitive displacement of native natural enemies</td>
<td>Nunney 2003</td>
</tr>
<tr>
<td>Genetic dilution of native species via hybridization with closely related species</td>
<td>Jolly 2000; Hopper et al. 2006</td>
</tr>
<tr>
<td>Changes in the balance of native species which lead to changes in predation pressure on other organisms</td>
<td>Baker et al. 2005</td>
</tr>
</tbody>
</table>

In general, retrospective studies have been conducted with a focus on those projects where population declines of non-target species have been observed or where the potential for non-target effects have been judged high, for example due to the polyphagous nature of the BCA (De Clercq et al. 2011). Even in these cases, serious non-target effects were not observed (Babendreier et al. 2003; Barron et al. 2003; Benson et al. 2003; van Lenteren et al. 2003; Barratt et al. 2006).

Unfortunately, there is definitely one BCA where increasing concerns about the adverse impact on non-target species have not only been raised but also materialized (van Lenteren et al. 2003; Majerus et al. 2006): the harlequin ladybird, *H. axyridis*. Declines (numerical and/or proportional) of certain native coccinellids, like *A. bipunctata*, have been observed in the invasive range of *H. axyridis* (Harmon et al. 2007; Adriaens et al. 2008; Brown et al. 2008a; De Clercq and Bale 2011; Evans et al. 2011; Roy et al. 2012). *Harmonia axyridis* appears to be displacing those native ladybirds with which it shares its habitat (Adriaens et al. 2008). The likely mechanisms are both intraguild competition and
predation based on the larger body size of *H. axyridis* and superior physical (*3*rd and *4*th larval instars have distinct spines) and chemical (alkaloid-laden secretions) defences in comparison with other ladybird species (Pell et al. 2008; Roy et al. 2008; Ingels and De Clercq 2011; Roy et al. 2012).
2.2 Environmental risk assessment

2.2.1 Regulatory framework

Various international frameworks control the (un)intentional introduction of exotic species from their native ranges to new environments (Loomans 2007). For example, the International Plant Protection Convention (IPPC) has developed International Standards for Phytosanitary Measures (ISPM) to prevent the spread of pests of plants and to promote appropriate measures for their control (IPPC 1997). Previously, legislation of IBCAs, when existent, usually fell under the responsibility of the national plant quarantine service focusing on plant protection (Wapshere 1974; Waage 1997). In 1992 the UN conference on Environment and Development formulated guiding principles resulting in the Convention of Biological Diversity (CBD), imposing all signing countries to prevent the introduction of alien species, to control and to eradicate those alien species which threaten local ecosystems. Since then more and more countries have put legislation in place concerning biological introductions that threaten biodiversity. The FAO ‘Code of Conduct for the Import and Release of Exotic Biological Control agents’ became an international standard as ISPM-3 (IPPC 1997). It provides procedures related to export, shipment, import and release of IBCAs and provides guidelines for risk management. However, methods for assessing environmental risks of IBCAs are only indirectly covered by existing standards on Pest Risk Analysis (ISPM-2 and ISPM-11) and novel strategies are needed for assessing and managing risks posed by IBCAs to biodiversity (Baker et al. 2005).

In Europe, various EC-directives have been adopted that control the introduction of exotic species of invertebrates, mainly those that may pose a threat to economically important plants (crops). However, none of these compulsory directives are specifically designed to control IBCAs, and are directed in general to preserve natural habitats or to prevent pests from entering or spreading in the EU. Besides, IBCAs are not covered by Directive 91/414/EEC or the new regulation 1107/2009/EC, imposing registration requirements for active ingredients of all plant protection products and
covering microbial organisms and semiochemicals (EU 1991, 2009). Until now the European Commission has not taken the initiative to develop regulation specifically targeting IBCAs although some initiatives are expected in the framework of a new regulation for invasive alien species (IAS). As a consequence, regulation of IBCAs across Europe is currently fragmented and evolving. Some countries have a well organized system (the Netherlands, Switzerland, Sweden, United Kingdom), others do not (Italy) and some are in the process of introducing regulation (France) (Bale 2011a). The situation in Belgium is even more complicated. While no regulation concerning BCAs is installed at the federal level, the introduction of exotic species is forbidden in Flanders by ‘het Soortenbesluit’, a 2009 regulation with the aim of protecting Flemish biodiversity. Exotic (= “non Flemish”) species can be released into the wild only if an environmental impact study showed no negative side-effects (Agentschap voor Natuur en Bos 2009). However, guidelines describing the methodology to conduct such an ERA are currently not available and at present it remains unclear who is responsible for evaluating the dossiers. A similar regulation exists in the Brussels region (Ordonnance Nature 2012) and is currently being developed in Wallonia.

Since 1996, when the European and Mediterranean Plant Protection Organization (EPPO) established its panel on ‘Safe use of biological control’, it has developed several standards on first import of exotic BCAs for research under contained conditions (PM 6/1(1)), import and release of exotic BCAs (PM 6/2(2)), as well as a list of IBCAs widely used in the EPPO region (EPPO 2001, 2002b, 2014). EPPO also has developed guidelines on Pest Risk Analysis, with a checklist of information required for making a PRA (EPPO 1997). This standard was brought in line with ISPM-11 (IPPC), but should be adopted for IBCAs specifically and does not yet provide working instructions for the risk assessment itself (EPPO 2002a). In 2003 the Organization for Economic Co-operation and Development (OECD) developed a Guidance for Information Requirements for Regulation of Invertebrates as Biological Control Agents, reviewing information that is required for risk assessment: characterization and identification of the organism, safety and effects on human health, environmental risks and efficacy, quality control and benefits of use. Also in 2003 the Council of the International Organization of
Biological Control-West Palaeartic Regional Section (IOBC-WPRS) appointed a Commission on Harmonization of Regulation of IBCAs (CHIBCA). Based on the FAO Code of Conduct, the EPPO standard and the OECD guidance, working groups drafted a detailed Guideline on Information Requirements for Import and Release of IBCAs in European countries (Bigler et al. 2005). A general methodology for risk assessment has been developed within the EU-financed project Evaluating Environmental Risks of Biological Control Introductions into Europe (ERBIC)(van Lenteren et al. 2003). The EU Policy Support Action, Regulation of IBCAs (REBECA), continued this review process with representation from all the main stakeholders - industry, regulators and researchers. The REBECA project produced a standardized application form to be used by companies when applying for a licence to release a non-native IBCA. The merit of this form is that it covers all types of application – native and non-native natural enemies, and ‘research only’ as well as release. A guidance document has been produced to accompany the application form which sets out the information that is required in each section. The form and guidance document have meanwhile been incorporated in EPPO standard 6/2 but it would be helpful if this form became the standard document to be used in all EU countries (Bale 2011a; Ehlers 2011).

2.2.2 Methodology for risk assessment

The European research project ERBIC attempted at formulating scientifically based guidelines to conduct risk assessments for (exotic) BCAs. This initiative had as a main conclusion that a risk assessment needs to be based on the establishment potential, host range and dispersal capacity of the candidate BCA and on the possible (in)direct effects this BCA may cause on non-target organisms. The flowchart designed by van Lenteren et al. (2006a) shows how abovementioned aspects of the risk assessment can be evaluated sequentially (Figure 1). First of all, a distinction between native and exotic BCAs should be made. When the BCA is native in the country where registration is requested, the procedure can be shortened and only possible (in)direct effects on non-targets need to be
studied. Although such effects of native natural enemies are generally believed to be transient as the population levels of native BCAs will be reduced under influence of their own natural enemies and other natural checks on their population growth, negative advice for release can be given when the risk of (in)direct effects of the BCA on non-targets is too extensive. The risk assessment for exotic BCAs is more extensive and depends on the goal of the release. If the natural enemy is to be released in a programme of classical biological control, establishment of the natural enemy is required *per se*. In this case the focus of the risk assessment will largely be on the host range of the natural enemy. When the host range of the BCA is limited to the target, it can be registered, while a natural enemy proven to have a large host range can be refused in this phase of the testing procedure or can be subjected to further testing. If the natural enemy on the other hand is to be released in a programme of commercial biological control by augmentation, the potential of the BCA to establish in our regions needs to be determined first. A species that cannot survive local winters (in temperate areas, like northwestern Europe) and that therefore is unlikely to cause permanent (in)direct non-target effects can immediately be granted for import and release and requires no further testing. In contrast, a species that is tolerant to lower temperatures and is likely to establish in our region can either be refused for registration or reconsidered for registration when the host range proves to be narrow. Thus, even polyphagous species qualify for registration when the overwintering and dispersal capacity is limited.

2.2.2.1 Establishment

The establishment of released natural enemies in a novel habitat depends on biotic (occurrence of suitable food) and abiotic (climate) factors. Although it has long been advocated that climate matching of the recipient system and the native range should help predict geographic spread (Louda et al. 2003; Cock et al. 2006), it is now apparent that climate matching may not be a sound basis for predicting long term survival and more comprehensive analysis of thermal tolerance is required (Hart
et al. 2002a, b). In the context of Europe, the influence of climate on establishment is mainly relevant to countries or regions within large countries that have a distinct winter season (Bale 2011a).

There are a number of indicators of an ability to establish in a temperate or colder climate and each of these can be assessed. The thermal budget refers to the accumulation of day degrees necessary to complete a generation and can be used to assess the number of generations that are theoretically possible per year. The lower developmental threshold, or base temperature, is the temperature below which no development occurs (Vannier 1994). Species originating from tropical and similar warm climates often have a high developmental threshold temperature, e.g. 10°C or higher, whereas temperate species can develop at 5°C or lower (Bale 2011a). When the total number of day degrees available in an area is below the minimum needed for a generation, establishment is unlikely (Vannier 1994; Boivin et al. 2006). Linked to this, when tropical species are placed in an ‘acclimation regime’, e.g. 5 of 10°C for seven days, a substantial proportion will die, whereas other species become more cold hardy over this period of time (Bale 2011a).

The occurrence of diapause (a dormant trait) is also a common feature in insects and mites living in temperate and cold climates, and where present in a candidate BCA, would increase the likelihood of winter survival and permanent establishment. For instance, a strain of the predatory mite *Neoseiulus californicus* (McGregor)(Acari: Phytoseiidae) collected in the UK was able to diapause, whereas strains found in the USA and Spain exhibited no or only a limited diapause propensity. A survey of overwintering refuges established that the strain from the UK was surviving field conditions (Jolly 2000). Diapause can either be obligatory (genetically programmed to occur at the same stage of development in every individual) or facultative (can be induced or averted depending on environmental cues). The most common diapause-inducing cue in arthropods is the decreasing photoperiod in autumn; there are standard regimes to test for diapause ability and these can be included routinely as part of the assessment of establishment potential (Bale 2011a).
Three laboratory testing protocols were designed to investigate the cold tolerance of exotic BCA: these determine the supercooling point (temperature at which body fluids freeze), lethal temperature (temperature at which 50% of the test organisms died) and lethal time (time at which 50% of the test organisms died at a temperature of 5°C) (Hatherly et al. 2005). A series of studies on a range of glasshouse agents has identified a strong correlation between lethal time at 5°C and the duration of survival in the field in winter (Figure 4). This relationship enables species to be categorized into three main ‘risk categories’: ‘low’, where all individuals die within 4-6 weeks in the field (like the predatory mites Amblyseius swirskii Athias-Henriot and Typhlodromips montdorensis (Schicha)(Acari: Phytoseiidae), the ladybird Delphastus calatinae (Horn)(Coleoptera: Coccinellidae), the predatory bug N. tenuis, and the parasitoid wasp E. eremicus), ‘medium’, where there is some survival after 2-3 months (like the predatory bug M. caliginosus), and ‘high’, where some individuals can survive for the entire winter (like the predatory bug Dicyphus hesperus Knight (Hemiptera: Miridae), and the predatory mite N. californicus)(Jolly 2000; Hart et al. 2002a, b; Tullett et al. 2004; Hatherly et al. 2004, 2005, 2008; Hughes et al. 2009). This relationship appears sufficiently robust for assessment to be based on laboratory studies alone (Bale 2011a).
2.2.2 Host range

Besides the exotic BCA’s capacity to survive local winters, its ability to locate and utilise wild hosts or prey is also an essential requirement for long-term survival (van Lenteren et al. 2003; Hatherly et al. 2005, 2009). The development of a concrete experimental methodology to assess host (or prey) range is still an ongoing process as several factors complicate this assessment, including the number of non-target species to be tested, the criteria for their selection, the need for reliable methods for determining acceptance of non-target species and the discrepancy between prey ranges observed in the field and prey ranges observed in the lab (Babendreier et al. 2005; Kuhlmann et al. 2006; van Lenteren et al. 2006b; Hatherly et al. 2009).

Despite these difficulties, it is now generally accepted that the development of a methodology to perform host range testing needs to be built on 2 pillars, the testing procedure and the selection of non-target organisms to test (Babendreier et al. 2005; van Lenteren et al. 2006b). Different experimental designs have been proposed: no-choice laboratory tests, choice laboratory tests, semi-
field tests and field tests. A no-choice test combines one or more organisms of a BCA with one or more organisms of a single test species in a closed arena (e.g. Petri dish or plastic container) under standard laboratory conditions. The strength of no-choice tests is that negative results are very robust and provide convincing evidence that a test species is not likely to be used as a host or prey in the field, which makes this test procedure popular with regulators. As a downside, a weak positive response to the test species may be artificially induced by confinement and a lack of choice, and the host range of BCA might be overestimated. However, an insect may in fact not have a choice of hosts if it expands geographically beyond the range of the target pest, if it invades habitats not occupied by the target pest, if the insect is partially out of synchrony with the target pest, or if the target pest is absent for any other reason (including biological control itself and chemical control) (Van Driesche and Murray 2004b).

In a choice situation, two or more non-target species are simultaneously presented to the BCA in a laboratory arena. The choice design is well suited to reveal if the agent shows a preference among potential host species and allows a more rapid examination of many species than possible if each must be studied separately. The weakness of this design is that preference for host A over B might erroneously be interpreted as B is not a host as it is hard to predict what will happen if no choice is available (Van Driesche and Murray 2004b).

In a semi-field test, multiple host plants with various non-target and target hosts are offered to the BCA in a large cage, creating a more natural situation (van Lenteren et al. 2006b). Finally, the open field test is an uncaged test outdoors. The test insects do not experience any unnatural influences that might alter their behavioural responses to potential hosts. This test can only be safely done in the targeted area of release if the BCA has a low cold tolerance and cannot establish, but can be an important last step before the release of the natural enemy under study (Van Driesche en Murray 2004b; van Lenteren et al. 2006b).
Within the EU-ERBIC project a sequential test scheme for determining host ranges of natural enemies was developed (Figure 5)(van Lenteren et al. 2003). In a first step non-target organisms are offered to the candidate BCA in a no-choice design. If none of the non-targets is attacked, one can stop testing as no direct effects on non-target species in the field are expected. If non-target species are attacked, one goes to step 2 to check whether the natural enemy attacks the non-target organism consistently. This behavioural test will determine if an increase in acceptance occurs after long-term exposure. If the non-target is not attacked at all or only at the end of the observation period, then the risk of direct effects on that species is small. In both cases, one can stop testing. For non-target species that are consistently attacked, one needs to go to the next step and determine host preference in a choice-design. The risk for the non-target is considered to be low when the species is not attacked or when the natural enemy does not develop a preference for the non-target host over the target after long-term exposure. The testing procedure can then be ended. When a shift in host preference occurs, one goes to step 4 and switches from a Petri dish as testing arena to a large cage equipped with host plants of the different non-target species. Non-targets not attacked in this experimental design are considered to be safe, whereas non-target species that are easily attacked on their host plant pose a high risk to suffer non-target effects. If possible (when the natural enemy has a low cold tolerance and cannot establish in the area of release or when tests can be done in the native range of the candidate BCA) the behaviour of the candidate BCA can be studied under natural circumstances in a field test. The risk for the non-target species can still be considered low when the species is not attacked under field conditions.
Figure 5: Sequential testing procedure for host range testing (after van Lenteren et al. 2003).
Several parameters can be used to characterize host acceptance of parasitoids and predators when studying their host or prey ranges. For predators, both adult and larvae are mobile and actively seek prey. Four processes can be observed: feeding (number of prey eaten per unit of time), adult survival (days), oviposition (numbers of eggs laid) and larval development (developmental time and survival, in days). For parasitoids, hosts are only found by adult females. The parasitisation process can be subdivided into several steps: host finding (upwind flight to herbivore/plant complex), host acceptance (parasitism rate) and regulation of host physiology (rate of encapsulation by the host) (Van Driesche en Murray 2004b).

A critical step in determining the host range of a natural enemy in the laboratory is the selection of the non-target species to be tested. van Lenteren et al. (2003) proposed a selection procedure for non-target species based on the phylogenetic centrifugal method used for the evaluation of weed BCAs. This procedure starts with testing non-target species that are closely related to the target and then progresses to species that are more distantly related to the target organism. If none of the non-target species is attacked, one can stop testing (Wapshere 1974; Lonsdale et al. 2001; van Lenteren et al. 2006b). However, the phylogenetic centrifugal approach may not always be feasible because of uncertainties in taxonomy and a much higher number of arthropod taxa compared to weeds (Kuhlmann et al. 2000) and the need to rear or collect sufficient numbers of the test species. Moreover, other parameters such as the feeding niche or the common habitat of target and non-target species may be meaningful (Messing 2001). Additional non-target species such as economically important species and threatened or aesthetic species may need to be tested as well (Sands and Van Driesche 2000; Babendreier et al. 2005; van Lenteren et al. 2006b). Within the EU-REBECA project a practical methodology for the selection of test species was proposed. In a first step, 3 non-target species would be tested, 2 species closely related to the target organism and 1 species that is less related to the non-target but that will frequently be encountered outdoors. If these tests show a host specificity, an additional 5 species would be tested in the second phase. The selection of
these species will be based on taxonomic relatedness, overlap in feeding niche, economic importance and potential status as endangered organism (Andermatt et al. 2007; Ehlers 2011).

Besides laboratory feeding and preference tests, making field observations of foraging predators and analyzing the gut contents of field-sampled predators could contribute to assessment of host ranges of candidate BCAs (Aebi et al. 2011).

2.2.2.3 Dispersal

The need to assess the dispersal capacity of a candidate BCA would be limited to specific circumstances. For example, for a glasshouse release with no establishment potential outdoors, there would be no need to assess dispersal. If unexpected establishment did occur, the species would most likely disperse over time and this would not be preventable. The situation where dispersal information is most valuable is for the mass release of large numbers of parasitoids or predators that even with an oligophagous feeding behaviour could impact on non-target fauna if dispersal from the release site was extensive (Bale 2011a, b). This type of study was conducted for the parasitoid Trichogramma brassicae Bezdenko (Hymenoptera: Trichogrammatidae) against the European corn borer Ostrinia nubilalis (Hübner)(Lepidoptera: Pyralidae) in Switzerland. It was shown that dispersal from the release sites in maize fields was extremely limited (Kuske et al. 2003).

Currently, there is no standard protocol to predict the dispersal capacity of natural enemies available, which is mainly due to its complex nature (i.e. the combination of long- and short-distance dispersal, the role of external factors in passive dispersal such as wind or transportation by man) and the practical difficulties to monitor and quantify dispersal. In the context of environmental impacts of commercial biological control, dispersal can be defined as the movement of natural enemies away from the release site and into the surrounding landscape. Mills et al. (2006) recommended mark-release-recapture (MRR) experiments as the best suited approach to assess the dispersal potential of BCAs. Such experiments analyse the density curves in relation to distance from a release point.
(Dobzhansky and Wright 1943). However, the strong influence of landscape matrix and climatic conditions on the outcome of the experiments were noted as important drawbacks of this strategy. Moreover, open field tests can only be done in the native range of the candidate BCA, as quarantine considerations prevent the test from being done into the intended area of introduction (Van Driesche and Murray 2004b).

2.2.2.4 (In)direct effects

When the BCA is native in the country where registration is requested or when it is released as a classical BCA and establishment is essentially irreversible, the study of (in)direct effects is an important part of an ERA.

As discussed above, there are three main areas in an ERA: establishment, host range and dispersal. These may interact to produce both direct and indirect effects (van Lenteren et al. 2006a). Examples of direct effects would include effects on non-target species, effects on other trophic levels (such as intraguild predation and plant feeding damage) and potential hybridization between the natural enemy and indigenous biotypes of closely related species. Further, the released BCA itself might be attacked by other organisms within the ecosystem (enrichment). This will have positive rather than negative effects on these populations, but it may indirectly have a significant impact on the original prey species of the top predator. A last direct effect may occur when the natural enemy acts as a vector for pathogens (vectoring)(van Lenteren et al. 2003; Bigler et al. 2006).

Indirect effects are those that occur when there is no direct interaction between the BCA and non-target species, such as competition of the released natural enemy with native natural enemies depending on the same prey species, which might in a worst-case scenario lead to competitive displacement. Another indirect effect may occur when the agent is an intraguild predator of another natural enemy and its suppression reduces predation pressure on its (usually herbivorous) prey
population. This may lead to temporal outbreaks of the herbivore (Rosenheim et al. 1995; van Lenteren et al. 2003; Messing et al. 2006).

Predation tests conducted in the laboratory can provide preliminary assessment of interactions among competing species. A second, powerful way to identify and analyse direct and indirect interactions in an arthropod community is to run experiments in large cages, to use surrogates (biologically close and realistic stand-ins for non-target species that are difficult to rear in the lab or for exotic natural enemies that cannot be imported due to quarantine measures), and to conduct surveys or manipulative experiments in the area of origin of a potential new natural enemy before its introduction. The principle behind this approach is to compare interactions at the level of the population (e.g. survival rates of natural enemies and herbivores) among treatments, including different combinations of natural enemies and herbivorous organisms. While risk cannot be eliminated, it can be managed with increasing confidence as our understanding of community dynamics grows incrementally (Messing et al. 2006).

### 2.2.3 Balancing risks and benefits

For polyphagous agents with establishment potential, companies should have the option to submit a dossier containing information on the risks and benefits of the proposed release compared with other possible controls. This information would be evaluated by the regulator as part of the ERA. If benefits outweigh risks and costs, the BCA may be approved, otherwise an application would be declined (Bale 2011b).

The proposed procedure for risk-benefit assessment consists of identifying, analysing and evaluating risks and benefits. First, costs and benefits of releasing the IBCA need to be identified and categorized as positive and negative effect on economy, human/animal health and environment (Table 2)(Bigler and Kölliker-Ott 2006).
Further, analysis involves determining the likelihood of assigned risks and benefits occurring, and the magnitude if they occur. Qualitative scales for probability (very unlikely → very likely) and magnitude (minimal → massive) have been described. A numerical value was added to each descriptor of probability and magnitude to quantify the risk/benefit. For each effect, the combination of probability and magnitude determines the level of risk associated with that effect (Loomans and van Lenteren 2006).

Table 2: Categories of costs and benefits of using IBCAs (Bigler and Kölliker-Ott 2006).

<table>
<thead>
<tr>
<th>Category</th>
<th>Costs</th>
<th>Benefits</th>
</tr>
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<tbody>
<tr>
<td><strong>Economy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Applicant/distributor</td>
<td>Development of a BCA</td>
<td>Sales of BCA, sustainable business</td>
</tr>
<tr>
<td>Farmer</td>
<td>Market price of a BCA and its application</td>
<td>Pest control with adequate efficacy, higher yield, product quality and revenue</td>
</tr>
<tr>
<td>Consumer</td>
<td>Higher prices and apparent lower quality of product</td>
<td>Lower prices and apparent higher quality of product</td>
</tr>
<tr>
<td>Society</td>
<td>BCA costs subsidized by government</td>
<td>Pest control with no/few risks to humans, animals and environment</td>
</tr>
<tr>
<td><strong>Human and animal health</strong></td>
<td>Allergies, stings/bites, nuisance</td>
<td>No hazards from pesticides</td>
</tr>
<tr>
<td><strong>Environment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil, water, air</td>
<td>No costs</td>
<td>Prevents pollution by pesticides</td>
</tr>
<tr>
<td>Biodiversity and ecosystems</td>
<td>Adverse effects on plants, animals, microorganisms and on ecosystem functions. Established species cannot be eradicated</td>
<td>Pest control with no/little effects on plants, animals, microorganisms and their functions. Replacement of control options with high impact on environment</td>
</tr>
</tbody>
</table>

The last step of a risk-benefit assessment involves balancing the risks against the benefits of releasing a BCA, compared to the current control methods. After the adverse and beneficial effects have been ranked, they need to be compared in order to evaluate whether the beneficial effects outweigh the adverse effects. Bigler and Kölliker-Ott (2006) suggested that it is best to start by comparing the risk and benefit with the highest rank. If the benefit with the highest rank exceeds the adverse effect.
with the highest rank, then the next step is to determine if the highest ranked benefit is greater than the combination of highest and second highest adverse effects. If, for example, the highest ranked beneficial effect is determined to be lower than the combination of the three highest ranked adverse effects, the second highest beneficial effect needs to be included in the comparison, and so forth, until all risks have been outweighed by beneficial effects (Bigler and Kölliker-Ott 2006).
2.3 Cryptolaemus montrouzieri

2.3.1 Taxonomy

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Animalia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
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<tr>
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<td>Superfamily</td>
<td>Cucuioidea</td>
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<td>Coccinellidae</td>
</tr>
<tr>
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<td>Scymninae</td>
</tr>
<tr>
<td>Genus</td>
<td>Cryptolaemus Mulsant</td>
</tr>
<tr>
<td>Species</td>
<td>Cryptolaemus montrouzieri Mulsant, 1853</td>
</tr>
</tbody>
</table>

Cryptolaemus montrouzieri Mulsant (Coleoptera: Coccinellidae) can be separated from all other known species of the genus by the coloration of its legs: it is the only species with dark tibiae on the meso- and metathoracic legs (Booth and Pope 1986; Kairo et al. 2012). Common names to refer to the species are the mealybug destroyer and Australian ladybird (Kishore et al. 1993; Hodek and Honěk 2009; Solangi et al. 2012).

2.3.2 Distribution

Cryptolaemus montrouzieri is indigenous to eastern Australia (Queensland, New South Wales) and the South Pacific Islands (Clausen 1978; Booth and Pope 1986), but has been imported into over 40 countries throughout the warm temperate and tropical regions of the world to control mealybugs and scales (Booth and Pope 1986; Funasaki et al. 1988; Kairo et al. 2012). In Europe, the first
introduction of *C. montrouzieri* as a classical BCA was made in 1908 in Italy. Subsequent releases were made in Spain (1926), Corsica (1970), France (1974) and Portugal (1984) (Roy and Migeon 2010; Kairo et al. 2012). Since 1992, *C. montrouzieri* is also being released in northwestern Europe as an augmentative BCA in protected cultivation and interior landscaping (Malais and Ravensberg 2002). Meanwhile, the ladybird has been reported outside greenhouses in natural environments in Sweden, Norway, Britain and Belgium (Ehnström and Lundberg 1997; Constantine and Majerus 1994; Hansen and Sagvolden 2007), but there is no unequivocal evidence of permanent establishment in those countries.

2.3.3 Biology

Like all species within Coccinellidae, *C. montrouzieri* is a holometabolous insect and undergoes a complete metamorphosis (Figure 6). The ladybird passes through three developmental stages (egg, larva and pupa) before reaching the adult stage. Survival, development and reproduction are regulated by biotic and abiotic factors (Malais and Ravensberg 2002).

![Figure 6: Life cycle of *C. montrouzieri* (source: www.bugsforbugs.com.au).](image-url)
2.3.3.1 Egg

The oval eggs are 1.5 mm long and have a pale yellow colour (Malais and Ravensberg 2002). Unlike other coccinellids, *C. montrouzieri* lays eggs singly or in small clutches of 3 to 4 eggs. The eggs are deposited among the cottony egg masses (ovisacs) of mealybugs (Figure 7). In this manner the female hides her eggs for ants tending the honeydew-excreting mealybug colony and avoids disturbance by ants. Besides, emerging larvae have a food source nearby which reduces egg cannibalism (Kaufmann 1996; Daane et al. 2007; Jayanthi et al. 2013).

![Figure 7: Large, pale yellow eggs of *C. montrouzieri* (indicated by black arrow) laid among the smaller, orange eggs of the mealybug *M. hirsutus* (source: www.nbaii.res.in).](image)

The incubation period of *C. montrouzieri* eggs takes 4-6 days at a temperature of 27°C, but prolongs to 6-7 days or 8-9 days when the temperature drops to 23°C and 21°C, respectively (Fisher 1963; Kaufmann 1996; Elsherif et al. 2010; Fand et al. 2010; Ghorbanian et al. 2011; Naser et al. 2011). The food source offered to *C. montrouzieri* affects the hatching rate of the eggs: the hatching rate decreased from 96% when the ladybirds were fed eggs of the citrus mealybug *Planococcus citri*
(Risso) (Hemiptera: Pseudococcidae) to 88% when they were provided with *E. kuehniella* eggs (Attia et al. 2011a).

![Figure 8: Adult (left), larva (top right) and pupa (bottom right) of *C. montrouzieri* (source: www.ozanimals.com (adult), www.scienceillustrated.com.au (larva), www.bugguide.net (pupa)).](image)

### 2.3.3.2 Larva and pupa

There are four larval stages separated by a moult. All larval instars have an elongated body shape and are covered in white waxy filaments, which provides camouflage among mealybugs and gives protection against ants (Figure 8) (Malais and Ravensberg 2002; Daane et al. 2007). Fourth instars can reach a body length of 13mm before pupating. As *C. montrouzieri* larvae feed not only on all stages of mealybugs, but also on their own eggs, larvae, pupae, and even on newly emerged adults, the last instars move away from still-feeding larvae and aggregate in small groups of 3-4 up to a dozen on non-infested plant parts (Kaufmann 1996). The adults spend about a day in the pupal case before emergence, in order to give the new cuticle time to harden (Malais and Ravensberg 2002).
Larval development depends on temperature and food (Table 3). Generally, developmental time decreases with increasing temperature (Babu and Azam 1987; Al-Humiari et al. 2011). When feeding on the hibiscus mealybug *Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae), *C. montrouzieri* development took 65.1 days at a temperature of 20°C and 46.7 days at 25°C (Babu and Azam 1987). Baskaran et al. (1999) found that *P. citri* was a more suitable prey for *C. montrouzieri* than the cochineal scale *Dactylopius tomentosus* (Lamark) (Hemiptera: Dactylopiidae) resulting in a faster developmental time. At 27°C, larval duration was 2, 3.9 and 4.3 days shorter when the ladybirds were reared on *E. kuehniella* eggs compared to their counterparts reared on the greenbug *Schizaphis graminum* (Rondani) (Hemiptera: Aphididae), the citrus mealybug *P. citri* and the solenopsis mealug *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae), respectively (Fand et al. 2010; Attia et al. 2011b).

### Table 3: Influence of temperature and food on larval developmental time (L1-adult) of *C. montrouzieri* (based on Kairo et al. 2012).

<table>
<thead>
<tr>
<th>Prey species</th>
<th>Temperature (°C)</th>
<th>Developmental time (days)</th>
<th>Survival (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudococcidae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phenacoccus madeirensis</em></td>
<td>17</td>
<td>60</td>
<td>34</td>
<td>Al-Humiari et al. 2011</td>
</tr>
<tr>
<td><em>Phenacoccus madeirensis</em></td>
<td>20</td>
<td>37.4</td>
<td>44</td>
<td>Al-Humiari et al. 2011</td>
</tr>
<tr>
<td><em>Maconellicoccus hirsutus</em></td>
<td>20</td>
<td>65.1</td>
<td>-</td>
<td>Babu and Azam 1987</td>
</tr>
<tr>
<td><em>Maconellicoccus hirsutus</em></td>
<td>25-28</td>
<td>46.5</td>
<td>-</td>
<td>Babu and Azam 1987</td>
</tr>
<tr>
<td><em>Phenacoccus solenopsis</em></td>
<td>27</td>
<td>31.1</td>
<td>-</td>
<td>Fand et al. 2010</td>
</tr>
<tr>
<td><em>Planococcus citri</em></td>
<td>27</td>
<td>30.8</td>
<td>94</td>
<td>Attia et al. 2011b</td>
</tr>
<tr>
<td><em>Planococcus citri</em></td>
<td>29</td>
<td>26.9</td>
<td>-</td>
<td>Baskaran et al. 1999</td>
</tr>
<tr>
<td><em>Dactylopiidae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dactylopius tomentosus</em></td>
<td>29</td>
<td>32.5</td>
<td>-</td>
<td>Baskaran et al. 1999</td>
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<td><em>Aphididae</em></td>
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<tr>
<td><em>Schizaphis graminum</em></td>
<td>27</td>
<td>28.1</td>
<td>58</td>
<td>Attia et al. 2011b</td>
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<tr>
<td><em>Pyralidae</em></td>
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<td></td>
</tr>
<tr>
<td><em>Ephestia kuehniella</em></td>
<td>27</td>
<td>26.1</td>
<td>62</td>
<td>Attia et al. 2011a</td>
</tr>
</tbody>
</table>
2.3.3.3 Adult

*Cryptolaemus montrouzieri* adults have a shortened oval body that is 3.8-4.6 mm long and 2.7-3.2 mm wide (Booth and Pope 1986). The head, pronotum, ten-segmented antennae and mouthparts are reddish yellow. The head has a smooth frons which is shining between punctures (Figure 8). The scutellum is black and strongly punctured. The elytra are shining black with a broad reddish yellow apical patch but without a metallic sheen (Booth and Pope 1986). Meso- and metathoracic legs are more or less pitchy to black, with paler tarsi. The colour of the femora and tibiae of the prothoracic legs is gender-dependent: those of the males are yellow, whereas those of the females are dominantly dark grey to black (Figure 9) (Fisher 1963; Booth and Pope 1986). A sex ratio of 1:1 is common (Kairo et al. 2012).

![Figure 9: Cryptolaemus montrouzieri male (left) and female (right) (photo: T. Segers).](image)

Females mate repeatedly throughout their life and may receive spermatozoa from 3 to 4 males. This frequent and multiple mating keeps the population genetically diversified (Kaufmann 1996; Hodek and Ceryngier 2000; Jayanthi et al. 2013). The females largely depend on mealybug wax filaments to stimulate oviposition and contact chemical cues seem predominant in inducing the search for an oviposition site. Besides, mechanical and thigmotactic cues, such as those provided by mealybug ovisacs, must be met (Merlin et al. 1996; Finlay-Doney and Walter 2012a). When no ovisacs are available, females refrain from ovipositing and withhold their eggs in their oviducts. After a long
absence of a suitable oviposition site, females will lay their eggs anywhere and immediately cannibalise them (Merlin 1992).

2.3.4 Biological control

The mealybug destroyer was one of the first coccinellids used in classical biological control and is nowadays intensively used for augmentation biological control in protected cultivation and interior landscaping (Chong and Oetting 2007; Muştu et al. 2008; Hodek and Honĕk 2009; Roy and Migeon 2010). Generally, C. montrouzieri has been regarded as an outstanding biological control success: of the 83 documented introductions, substantial control of the target pest was achieved in 14 cases and the predator became either permanently or temporarily established in 37 and 10 cases, respectively (Moore 1988; Kairo et al. 2012). Only in 19 instances, C. montrouzieri failed to control the target pest, which was contributed to negative effects of pesticides (Samuel et al. 1981; Meyerdirk et al. 1982; Hassan et al. 1983) and inadequate prey numbers (Clausen 1978; Oncuer and Bayhan 1982).

Cryptolaemus montrouzieri is mainly released against mealybug pests (Figure 10). Within the Pseudococcidae family, the citrus mealybug P. citri is the most cosmopolitan species found in all (sub)tropical regions. In northwestern Europe, this mealybug is a pest of citrus, cucumber, egg plant, melon and ornamental plants in greenhouses (Clausen 1978; Gill and Sanderson 1998; Malais and Ravensberg 2002; Demirci et al. 2011). Mealybugs damage plants by feeding on the phloem. Their honeydew production leads to the growth of sooty molds, which block photosynthesis and contaminate fruits (Malais and Ravensberg 2002; Kerns et al. 2004). Mealybugs also transmit viruses (Clausen and Bartlett 1978; Cabaleiro and Segura 1997; Blumberg and Van Driesche 2001; Cid et al. 2007).
Control of mealybugs with insecticides is difficult because of the mealybugs’ ability to hide in inaccessible places of the plant (like the leaf axis, leaf sheats, between twining stems and under loose bark) and to secrete thick layers of protective wax (Figure 10) (Demicri et al. 2011). Besides, mealybugs quickly develop resistance to pesticides and the frequent application of chemical products is detrimental to natural enemies (Flaherty et al. 1982). Therefore, the biological control by parasitoids and predators represents an important method of controlling these pest insects (Malais and Ravensberg 2002; Ghafoor et al. 2011).

*Cryptolaemus montrouzieri* has also been reported to feed on a wide range of other hemipterans in the field, including aphids, scales and whiteflies (Figure 11) (Malais and Ravensberg 2002; Ślipiński 2007; Finlay-Doney and Walter 2012b; Kairo et al. 2012; Cock 2013). Its effectiveness in suppressing populations of these alternative prey, however, has rarely been studied (Finlay-Doney and Walter 2012b).
Figure 11: *Cryptolaemus montrouzieri* adult feeding on mealybugs (left), *C. montrouzieri* larva feeding on an aphid (right) (source: www.organicgardeninfo.com, www.icmag.com).

### 2.3.5 Rearing procedures

*Cryptolaemus montrouzieri* is traditionally produced using mealybug hosts, primarily *P. citri* and *M. hirsutus*, reared on plant materials like potato sprouts (Fisher 1963) or pumpkins (Babu and Azam 1987; Kishore et al. 1993). Commercial insectaries still employ the methods described by Fisher (1963) for mass production of *C. montrouzieri* (Hodek and Honěk 2009). Mealybugs are reared in the dark on bleached (etiolated) potato sprouts, stacked in trays in an open room. They are allowed to develop for 20-25 days, after which adult ladybirds are introduced. Newly emerged coccinellids, which are attracted to light, are collected by opening screened windows and scooping them off the mesh of the cages using a broad scoop that narrows into a funnel connected to a plastic tube. Several million *C. montrouzieri* can be produced yearly using these methods (Kairo et al. 2012).

For smaller-scale cultures, Kishore et al. (1993) found that the best yields were obtained when pumpkins were placed on a plastic stand in wooden, glass-topped cages. Pumpkins were infested with a minimum of 50 gravid female *M. hirsutus* and the resulting 20-day-old colony was exposed to 10 ovipositing female *C. montrouzieri* for 10 days. An average of 250 ladybirds was obtained per pumpkin 50-55 days after initial infestation with mealybugs.

Not only are these natural rearing methods time-consuming but the seasonal availability of these plant materials also complicates the continuous supply of adequate quantities of mealybugs. In order
to improve the economy of mass production, factitious food sources have been employed for the rearing of different coleopteran predators with notable success (Hodek and Honěk 2009; Riddick and Chen 2014). For example, eggs of different Lepidoptera species have shown to be a suitable factitious food for the predatory ladybirds Adalia bipunctata (L.)(De Clercq et al. 2005), Propylea japonica (Thunberg)(Hamasaki and Matsui 2006) and Harmonia axyridis (Pallas)(Berkvens et al. 2008b). Cryptolaemus montrouzieri larvae can also be successfully reared on eggs of the Angoumois grain moth Sitotroga cerealella (Olivier)(Lepidoptera: Gelechiidae) and the Mediterranean flour moth Ephestia kuehniella Zeller (Lepidoptera: Pyralidae). When using these lepidopteran eggs as prey, it was found necessary to provide mealybug ovisacs in order to induce oviposition (Pilipjuk et al. 1982; Attia et al. 2011a). Therefore, the implementation of factitious prey in the mass culture of C. montrouzieri largely depends on the development of an artificial oviposition substrate that triggers oviposition in the absence of mealybug ovisacs. To our knowledge no artificial oviposition substrate has been reported in the literature for C. montrouzieri.
2.4 *Macrolophus pygmaeus*

2.4.1 Taxonomic classification and identification

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Animalia</th>
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<tbody>
<tr>
<td>Phylum</td>
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<td>Dicyphina</td>
</tr>
<tr>
<td>Species</td>
<td><em>Macrolophus pygmaeus</em> Rambur, 1839</td>
</tr>
</tbody>
</table>

Despite the crucial importance of correct species identification of a natural enemy for the outcome of a biocontrol program, much of the literature on *Macrolophus* spp. remains confused due to taxonomic problems. van Lenteren (2003) reported that *Macrolophus caliginosus* Wagner (Hemiptera: Miridae) is the predatory insect most commonly sold by commercial biocontrol companies. However, because of a lack in consistency of morphological traits of species within the genus *Macrolophus*, questions about the correct identity of the marketed species have arisen. Molecular techniques revealed that the species sold by some European biocontrol companies as *M. caliginosus* was in fact *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae)(Perdikis et al. 2003; Martinez-Cascales et al. 2006; Machtelinckx et al. 2009).
2.4.2 Distribution

*Macrolophus pygmaeus* is widely distributed in Europe and has been reported from the Mediterranean Region up to Finland (Kerzhner and Josifov 1999; Fauna Europaea 2011). The species has also been recorded in Auckland, New Zealand by Eyles et al. (2008) but there is no further evidence of its establishment there. The population that became widely commercially available for biological control in protected cultivation in Europe originates from the Mediterranean region (J. Klapwijk, pers. comm.).

2.4.3 Biology

Mirids are hemimetabolous insects with an egg stage, five nymphal instars and an adult stage. Hemimetabolous insects undergo an incomplete metamorphosis and the nymphs resemble the adults but are smaller and lack certain structures such as wings and genitals (Gullan and Cranston 2005). Development of *M. pygmaeus* is affected by biotic and abiotic factors such as temperature, food source and host plant (Perdikis and Lykouressis 1997, 2000, 2002; Hommes and ter Horst 2002).

2.4.3.1 Egg

The banana-shaped eggs of *Macrolophus* spp. are approximately 1mm in size, pale-coloured and are enclosed by a thin transparent membrane (Hillert et al. 2002). Females lay their eggs deep in the plant tissue with only the operculum remaining visible (Fauvel et al. 1987). This operculum contains respiratory horns (aeropyles) which allow gas exchange between the egg and the environment (Figure 12)(Constant et al. 1994). The incubation time in optimal conditions (25°C) is 11 days (Fauvel et al. 1987).
2.4.3.2 Nymph

*Macrolophus pygmaeus* undergoes five moults before the adult stage. Wing development is external and the so-called 'wing pads', located dorsally on the meso- and metathorax, enlarge with every moult (Figure 13) (Hillert et al. 2002). While the wing pads of third instar nymphs can only be visualized with a microscope, these of the fourth instars are further developed, cover a part of the abdomen and can be observed with the naked eye. During the fifth nymphal stadium, the wing pads remain relatively constant in size, but the size of the abdomen increases, resulting in a decrease of the covered abdominal area (Vandekerkhove 2010).

Generally, the nymphal developmental time of *M. pygmaeus* decreases with increasing temperature. The lower temperature threshold of these bugs was calculated to be 9.19°C (Perdikis and Lykouressis 2002), whereas a temperature of 32.5°C was estimated to be the upper temperature threshold (Hommes and ter Horst 2002). Also food source offered to the nymphs can have a significant impact on their developmental time. Perdikis and Lykouressis (2000) found that the whitefly *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) was a more suitable prey for *M. pygmaeus* than the peach aphid *Myzus persicae* (Sulzer) and the potato aphid *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae). A third factor that can play a role in the rate and success of nymphal
development is the host plant. Of several host plants tested egg plant *Solanum melongena* L. appears the most suitable for nymphaal development of *M. pygmaeus* (Perdikis and Lykouressis 1997, 2000).

![Figure 13: Overview of the five nymphaal instars of *M. pygmaeus* (source: Hillert et al. 2002).](image)

2.4.3.3 Adult

*Macrolopolus* adults have a slim body, long legs, vary between 3.3 and 3.9 mm in size and are yellowish-green in colour. Their head is pentagonal, usually with dark longitudinal stripes running from the eyes to the pronotum (Goula et al. 2002). The sexes can be easily distinguished by the shape of the abdomen. While females have a swollen abdomen with a clearly visible brown ovipositor at the ventral side (Figure 14), the abdomen of males is flat. The mating of *Macrolopolus* males and females occurs end to end, with the male directly across the female (Figure 14). The female usually only mates with one male and receives enough sperm to fertilize all of her eggs (Franco et al. 2011).

Several European populations of *Macrolopolus pygmaeus* are infected with the endosymbiotic bacterium *Wolbachia pipientis*, which induces cytoplasmic incompatibility and causes an abnormal reproductive pattern (very low egg hatching rate) when an infected male mates with an uninfected
female or a female infected with a different *Wolbachia*-strain (Machtelinckx et al. 2009). Besides *Wolbachia*, the endosymbionts *Rickettsia limoniae* and *R. bellii* were also detected in the ovaries of *M. pygmaeus* females. However, the impact of the *Rickettsia* species on the biology of *Macrolophus* bugs is not fully understood (Machtelinckx et al. 2012).

![Image of M. pygmaeus](image)

*Figure 14*: Adult female (left) and a mating pair of *M. pygmaeus* (right) (source: Koppert (left), B. Vandekerkhove, V. De Puyssellery and U. Wyss (right)).

### 2.4.4 Biological control

*Macrolophus pygmaeus* is a voracious predator that is mainly used in augmentative biological control of the whitefly pests *T. vaporariorum* and *Bemisia tabaci* (Gennadius)(Hemiptera: Aleyrodidae) in protected cultivation in Europe. Both whitefly larvae and adults feed on the phloem of host plants and cause either direct damage, by depleting nutritional components, and indirect damage, as a result of sooty molds that grow on honeydew excreted by the insects as they feed (Figure 15). The sooty molds block photosynthesis in leaves and contaminate fruits (e.g. tomatoes, eggplants), which become unmarketable (Figure 4). Furthermore, as vectors of viral diseases, even low densities of whiteflies can have negative economic consequences for the grower (Oliveira et al. 2001; Jones 2003). Since many whitefly populations are resistant against carbamates, pyrethroids, organophosphates and neonicotinoids, biological control is considered to be the best mechanism to suppress the whiteflies (Alomar et al. 2006).
Figure 15: Whitefly adult (left) and whitefly damage to tomato (right) (source: Koppert (left), D. Blancard (INRA) (right)).

Because *M. pygmaeus* has a long generation time and it takes up to two months for the population to build up, it is important to introduce the predator early in spring, which allows the bugs to get established in the crop. During this period, the use of other natural enemies, such as the parasitoids *Encarsia formosa* (Gahan) or *Eretmocerus eremicus* Rose and Zolnerowich (Hymenoptera: Aphelinidae), is required to suppress the whitefly populations below economic levels (Biobest 2014).

Besides whiteflies, the predator also contributes to the control of thrips, aphids and spider mites and is a key BCA of the tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Perdikis and Lykouressis 2000; Urbaneja et al. 2009). The latter pest insect has invaded tomato crops in Europe causing significant economic losses. *Macrolophus pygmaeus* shows a high predation potential against eggs and small larvae of the leafminer. In tomato fields where *M. pygmaeus* populations were established, damage caused by *T. absoluta* was significantly lower than in fields treated with pesticides (Urbaneja et al. 2009).

### 2.4.5 Rearing procedures

The augmentative use of *M. pygmaeus* requires large numbers of commercially produced insects of high quality (i.e., good developmental performance, high reproductive fitness and high predation capacity). The relatively high cost price of commercial natural enemies is an important drawback of
augmentative biological control and slows down its adoption in most agricultural systems (van Lenteren 2012). This obstacle can be in part overcome by rationalizing the mass production techniques for arthropod natural enemies thus lowering their production cost (van Lenteren and Tommasini 2003). In many cases, natural enemies are reared on their natural prey, which are, in turn, maintained on their host plants. Maintaining three trophic levels creates high labour costs and costs associated with operating and maintaining separate equipment and space. Additionally, the more trophic levels, the greater the risk of mishaps at one of these levels, that could jeopardize the rearing system (De Clercq 2008; Riddick 2009; De Clercq et al. 2014).

In order to improve the economy of mass production, factitious (unnatural) food sources have been employed for the rearing of different heteropteran predators with notable success (De Clercq et al. 2014). For example, eggs of the flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) have shown to be a suitable factitious food for the predatory bugs *Orius laevigatus* (Fieber)(Hemiptera: Anthocoridae), *M. caliginosus* and *M. pygmaeus* (Arijs and De Clercq 2001; Bonte and De Clercq 2008; Fauvel et al. 1987; Castañe et al. 2006; Vandekerkhove et al. 2006, 2009). Fauvel et al. (1987) and Cocuzza et al. (1997) found that *M. caliginosus* and *O. laevigatus*, respectively, performed even better on *E. kuehniella* eggs than on some of their natural prey. Since the 1990s, lepidopteran eggs have become the standard food for commercially produced heteropteran predators (Fauvel et al. 1987; Castañe et al. 2006).

A possible next step in rationalizing the rearing system for *M. pygmaeus* is the development of an artificial diet (De Clercq et al. 2013). Vandekerkhove et al. (2006) investigated whether artificial diets based on hen’s egg yolk could provide adequate nutrition for the development and reproduction of *M. pygmaeus*. They reported that the artificial diets, encapsulated in Parafilm domes, resulted in prolonged development and lower adult weights as compared with *E. kuehniella* eggs, but survival was similar. Further, dissection of females at day 7 indicated that females fed *Ephestia* eggs had more developing eggs (oocytes) in their ovaries than those fed an artificial diet.
Chapter 3

An inventory of exotic biological control agents used in Europe

Redrafted after:

Chapter 3

3.1 Introduction

In commercial biological control both indigenous and exotic natural enemies are used to control pest populations. Concerns about the potential negative impact of exotic BCAs on the local environment create the need to regulate the import and use of exotic species (van Lenteren et al. 2003; Loomans and van Lenteren 2005; De Clercq et al. 2011). As a first step to support regulatory initiatives in Belgium within the framework of the Macoreg project funded by the Federal Belgian authorities invertebrate natural enemies that are currently commercially available to Belgian growers were inventoried. As growers in Belgium have access to the European market, the inventory was in essence European in scale. The species were then classified as either indigenous or exotic to Belgium (as a legal entity) and the neighbouring countries (as part of an ecozone approach). The results of this inventory are presented and discussed below.

3.2 Materials and Methods

To inventorize the IBCAs that are commercially available in Belgium, an internet search was conducted and the products of 16 European companies active in the production of natural enemies were listed. The main goal of this survey was to list those BCA that were anno 2012 commercially available to Belgium growers. In contrast to earlier surveys (Cock et al. 2010, van Lenteren 2012), our inventory was limited to the presently marketed products of commercial companies and did not include natural enemies reared for research purposes at universities and research institutes, natural enemies which had been released in the past or species used only very occasionally. We consulted the websites of all commercial producers of BCAs in Europe which we were able to find online (Table 4) and included their products in the inventory. If needed, additional information was requested from the producers by email.
Table 4: List of companies active in the production of natural enemies that were included in the inventory.

<table>
<thead>
<tr>
<th>Commercial producer of BCA</th>
<th>Country of headquarter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andermatt BioControl AG</td>
<td>Switzerland</td>
</tr>
<tr>
<td>Biobest NV</td>
<td>Belgium</td>
</tr>
<tr>
<td>Bioplanet s.c.a.</td>
<td>Italy</td>
</tr>
<tr>
<td>Borregaard Bioplant ApS</td>
<td>Denmark</td>
</tr>
<tr>
<td>Biotop</td>
<td>France</td>
</tr>
<tr>
<td>BCP Certis</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>E-nema</td>
<td>Germany</td>
</tr>
<tr>
<td>Entocare CV</td>
<td>The Netherlands</td>
</tr>
<tr>
<td>Intrachem Bio Italy SpA</td>
<td>Italy</td>
</tr>
<tr>
<td>Katz Biotech AG</td>
<td>Germany</td>
</tr>
<tr>
<td>Koppert BV</td>
<td>The Netherlands</td>
</tr>
<tr>
<td>Neudorff GmbH</td>
<td>Germany</td>
</tr>
<tr>
<td>Nijhoff BGB</td>
<td>The Netherlands</td>
</tr>
<tr>
<td>Nueztlinge Sautter &amp; Stepper</td>
<td>Germany</td>
</tr>
<tr>
<td>Syngenta Bioline</td>
<td>United Kingdom</td>
</tr>
</tbody>
</table>

Information on the original distribution (place of origin) of the IBCA was collected by consulting the online databases of Fauna Europaea, DAISIE (Delivering Alien Invasive Species Inventories for Europe) and EPPO (European and Mediterranean Plant Protection Organization). Practical problems were faced related to incomplete information found in the databases as not every country has historical records on presence and distribution of all species under study. The databases depend on information gathered by experts. When the species’ distribution was never explored in one country but its presence/absence was confirmed in one or more neighbouring countries, a fragmented view of the distribution of this species appears. When distribution data for Belgium were lacking and an organism was classified as indigenous in one of our neighbouring countries with a similar climate (The Netherlands, Germany, (Northern)-France), the natural enemy was considered native to Belgium.


### 3.3 Results and Discussion

More than 90 invertebrate species are currently commercially available in Europe: insects represent approximately 80% of these species, predatory mites 14% and entomopathogenic nematodes 6% (Appendix 1). Within the Insecta, the Hymenoptera (43 species) and the Coleoptera (17 species) deliver most species, followed by the Hemiptera (8 species)(Table 5). Well-known and worldwide used representatives of these groups are the parasitoids *Encarsia formosa* (Gahan), *Eretmocerus eremicus* Rose and Zolnerowich and *Aphidius colemani* Viereck (Hymenoptera: Aphelinidae), the ladybird *Cryptolaemus montouzieri* Mulsant (Coleoptera: Coccinellidae) and the predatory bug *Macrolelophus pygmaeus* Rambur (Hemiptera: Miridae). Although the predatory mites account for only 14% of all species commercially available on the Belgian market, they provide important tools in the control of whiteflies, thrips and mites, including *Amblyseius cucumeris* (Oudemans), *Phytoseiulus persimilis* Athias-Henriot and a more recent addition, *Amblyseius swirskii* (Athias-Henriot)(Acari: Phytoseiidae).

The actual number of species available in Europe is probably higher than estimated here. Cock et al. (2010) noted that 170 species of invertebrate natural enemies are used in augmentative biological control in Europe and van Lenteren (2012) reported a number of no less than 230 species worldwide. Although the number of available species for augmentation biological control is huge, Cock et al. (2010) and van Lenteren (2012) calculated that only about 25 biocontrol agents make up for 90% of the market value and that the majority of the listed species are only sold in numbers of hundreds to thousands of individuals per week.
About 53% of the IBCAs which are commercially available to Belgian growers were found to be non-native to Belgium or neighbouring countries (The Netherlands, Germany, (Northern)-France)(Table 5). Approximately 40% of these alien species is from non-European origin and more or less 15% is used outside its original distribution in Europe. The proportion of non-native species among insects and predatory mites is about the same (60%) but all nematodes are (presumably) indigenous. Among the exotic species, economically important natural enemies are found, including E. formosa, E. eremicus, P. persimilis and certain Amblyseius species.

van Lenteren (2012) reported that until the year 2000 more new exotic species were commercialized in Europe than indigenous species (77 versus 58), but that this trend has changed and that nowadays more indigenous species are marketed than exotic species (18 versus 6). Not only are indigenous natural enemy species increasingly evaluated for first use, but also 9 species of exotic BCAs have been replaced by indigenous species. An important example is the replacement of the North American predatory bug Orius insidiosus (Say) by Orius laevigatus (Fieber)(Hemiptera: Anthocoridae)(van Lenteren 2012). This shift is not only caused by the increasing concerns about the risks associated with the import and release of exotic BCAs and the resulting registration demands for non-native IBCAs, but is also related to practical problems ensuing from recent international agreements on the access and benefit sharing of native biodiversity (Cock et al. 2010).
The original distribution of the BCAs that are not native to Belgium or surrounding areas is shown in Figure 16. Except for South America (5%), all continents deliver more or less 20% of the exotics. The European natural enemies that have not previously been described from Belgium and surrounding areas mostly have a Mediterranean origin.

![Figure 16: Original distribution of the IBCAs that are not native to Belgium or neighbouring areas.](image)

The study indicated that information on the original distribution of natural enemies is often incomplete. Moreover, questions can be raised about the feasibility of considering a small geographical area like Belgium or even Flanders (see "Soortenbesluit") as an ecologically relevant entity. The development of a harmonised regulation among different countries of a similar ecoregion is therefore recommendable (Cock et al. 2006).
Chapter 4

A semi-artificial rearing system for *Cryptolaemus montrouzieri*

Redrafted after:

4.1 Introduction

The relatively high cost price of commercial natural enemies is an important drawback of augmentative biological control and slows down its adoption in most agricultural systems (van Lenteren 2012). This obstacle can be in part overcome by rationalizing the mass production techniques for arthropod natural enemies by replacing natural rearing systems (i.e. using the natural prey and its host plant) with production systems using unnatural foods and artificial oviposition substrates and thus lowering their production cost (van Lenteren and Tommasini 2003).

Lepidopteran eggs are a commonly used unnatural diet for the production of various insect predators, including predatory ladybirds (De Clercq et al. 2005; Hamasaki and Matsui 2006; Berkvens et al. 2008b; Riddick and Chen 2014). Whereas in natural rearing systems the ladybird Cryptolaemus montrouzieri Mulsant (Coleoptera: Coccinellidae) is produced on mealybugs reared on potatoes or pumpkins, some studies showed that its larvae can also be successfully reared using eggs of the Angoumois grain moth Sitotroga cerealella (Olivier) (Lepidoptera: Gelechiidae) and the Mediterranean flour moth Ephestia kuehniella Zeller (Lepidoptera: Pyralidae) as unnatural prey (Pilipjuk et al. 1982; Attia et al. 2011a).

However, previous studies indicated that C. montrouzieri females largely depend on mealybug wax filaments to stimulate oviposition and that contact chemical cues seem predominant in inducing the search for an oviposition site. Besides, mechanical and thigmotactic cues, such as those provided by mealybug ovisacs, must be met (Merlin et al. 1996; Finlay-Doney and Walter 2012a). Therefore, the implementation of factitious prey in the mass culture of C. montrouzieri is only useful if an artificial oviposition substrate is available that triggers oviposition in the absence of mealybug ovisacs. To our knowledge no artificial oviposition substrate has been reported in the literature for C. montrouzieri.

In this chapter, the developmental and reproductive performance of the predator reared on E. kuehniella eggs as a food source and synthetic wadding as an oviposition substrate was compared to that in a natural rearing method based on the citrus mealybug Planococcus citri (Risso) (Hemiptera:}
Pseudococcidae) as prey and *P. citri* ovisacs as a substrate for oviposition. Because of the relatively high cost price of *E. kuehniella* eggs, we also tested the nutritional value of frozen bee pollen to sustain the development and reproduction of *C. montrouzieri* and investigated whether the production cost of the predator can be lowered further by mixing the flour moth eggs with bee pollen.

### 4.2 Materials and Methods

#### 4.2.1 Insect cultures

Laboratory colonies of *C. montrouzieri* and *P. citri* were established in 2010 with larvae and nymphs acquired from Katz Biotech AG (Baruth, Germany) and from Koppert BV (Berkel and Rodenrijs, The Netherlands), respectively. The mealybugs were cultured on etiolated potato sprouts and kept in a dark room at ambient conditions. Potatoes infested with mealybugs and covered with ovisacs were transferred to the stock colony of *C. montrouzieri* maintained at 25±1°C, 75 ± 5% relative humidity (RH) and a 16:8h (L:D) photoperiod.

#### 4.2.2 Experimental set-up

In a first laboratory experiment, the potential of *E. kuehniella* eggs as a food source and the suitability of Rolta®Soft synthetic polyester wadding (1x1cm) as an artificial oviposition substrate for *C. montrouzieri* (Semi-artificial rearing method 1) was tested. *Ephestia kuehniella* eggs were offered to larvae and adults in a 1.5cm (Ø) plastic dish. Developmental parameters (immature survival, developmental time, adult body weight), reproductive traits (preoviposition period, total oviposition, egg hatch), sex ratio and longevity of predators reared under these conditions were compared to those of a control group offered *P. citri* nymphs as a diet and mealybug ovisacs as an oviposition substrate (Natural rearing method). Because oviposition may be triggered by the presence of food
for the developing larvae in the oviposition substrate, a treatment in which the females were offered polyester wadding sprinkled with *E. kuehniella* eggs in addition to the moth eggs supplied in a plastic dish was also included (Semi-artificial rearing method 2).

In a second experiment, four food sources were tested. The value of frozen moist bee pollen as a (supplemental) food source to sustain the development and reproduction of *C. montrouzieri* was assessed. The performance of the ladybird on a mixture of *E. kuehniella* eggs and bee pollen (1:1 proportion)(Food source 1) and on bee pollen alone (Food source 2) was compared with that on *P. citri* nymphs (Food source 3) or *E. kuehniella* eggs (Food source 4). Both flour moth eggs and bee pollen were supplied by Koppert BV (Berkel and Rodenrijs, The Netherlands). Polyester wadding served as an oviposition substrate in all treatments, including the mealybug treatment. Table 6 summarizes the experimental set-up of both Experiment 1 and 2.

**Table 6:** Overview of the experimental treatments to assess the suitability of the semi-artificial rearing method for *C. montrouzieri* (Experiment 1) and to evaluate the value of different alternative food sources for rearing *C. montrouzieri* (Experiment 2).

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Food source (during development and reproduction)</th>
<th>Oviposition substrate</th>
<th>Time period during which reproduction was followed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural rearing method</td>
<td><em>P. citri</em> nymphs</td>
<td>Mealybug ovisacs</td>
<td>Entire lifetime females</td>
</tr>
<tr>
<td>Semi-artificial method 1</td>
<td><em>E. kuehniella</em> eggs</td>
<td>Polyester wadding</td>
<td>Entire lifetime females</td>
</tr>
<tr>
<td>Semi-artificial method 2</td>
<td><em>E. kuehniella</em> eggs</td>
<td>Polyester wadding sprinkled with <em>E. kuehniella</em> eggs</td>
<td>Entire lifetime females</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Food source</th>
<th>Food source (during development and reproduction)</th>
<th>Oviposition substrate</th>
<th>Time period during which reproduction was followed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food source 1</td>
<td><em>E. kuehniella</em> eggs + bee pollen</td>
<td>Polyester wadding</td>
<td>1 month after first egg</td>
<td></td>
</tr>
<tr>
<td>Food source 2</td>
<td>Bee pollen</td>
<td>Polyester wadding</td>
<td>1 month after first egg</td>
<td></td>
</tr>
<tr>
<td>Food source 3</td>
<td><em>P. citri</em> nymphs</td>
<td>Polyester wadding</td>
<td>1 month after first egg</td>
<td></td>
</tr>
<tr>
<td>Food source 4</td>
<td><em>E. kuehniella</em> eggs</td>
<td>Polyester wadding</td>
<td>1 month after first egg</td>
<td></td>
</tr>
</tbody>
</table>

In all treatments of both experiments, newly emerged first instar larvae (± 50 larvae in both experiments depending on the availability of ladybird larvae and mealybugs) were taken out of the stock colony and placed individually in polystyrene Petri dishes (Ø: 9 cm, height: 2 cm). All foods used in both experiments were offered *ad libitum* and replenished every other day. Water was provided to larvae and adults by way of a moist wadding plug fitted into a 1.5cm (Ø) plastic dish. Survival and
development of *C. montrouzieri* larvae was monitored daily. Newly emerged adults were sexed and weighed using a Sartorius Genius ME215P balance, after which they were randomly paired. Adults received the same food as during their larval stage. Oviposition substrates were checked daily for eggs to determine the preoviposition period. Once the first egg was laid substrates were replaced three times a week for a time period of 1 month (second experiment) or until the female died (first experiment). Oviposition rate and egg hatch were monitored during the first 30 days of egg laying (second experiment) or during the entire lifetime of the female (first experiment). Longevity of both males and females was determined. All experiments were conducted in a climatic chamber set at 25±1°C, a relative humidity of 75±5% RH and a 16:8h (L:D) photoperiod.

4.2.3 Statistical analysis

All data were analyzed using SPSS 21.0 (SPSS Inc. 2009). In both experiments, survival rates, egg hatch rates and sex ratios were considered as binary data, which enabled us to calculate standard errors on percentages. The means were compared by means of a logistic regression. This regression is a generalized linear model using a probit (log odds) link and a binomial error function (McCullagh and Nelder 1989). P-values below 0.05 were considered significant.

For the first experiment, the developmental parameters of predators reared according to the two semi-artificial systems were pooled as the larvae received the same treatment. A Kolmogorov-Smirnov test indicated that developmental time and adult body weight of naturally and semi-artificially reared individuals were normally distributed. These parameters were analyzed using Student’s homo- or heteroscedastic t-tests when the Levene test indicated equal or unequal variances, respectively. The preoviposition period and longevity parameters were also normally distributed and analyzed using a one-way analysis of variance (ANOVA); means were separated using Tukey’s test as a Levene test proved homoscedasticity. A non-parametric Kruskal-Wallis H test was used to evaluate differences in the number of deposited eggs among treatments.
For the second experiment, a Kolmogorov-Smirnov test again indicated that adult body weight and total number of deposited eggs were normally distributed; these parameters were therefore analyzed using a one-way ANOVA and means were separated using Tukey’s test, after homoscedasticity was confirmed using a Levene test. Developmental times and preoviposition periods were not normally distributed and thus analyzed using a Kruskal-Wallis H test followed by Mann-Whitney U tests (SPSS Inc. 2009).

### 4.3 Results

Predator larvae reared in the first experiment on *E. kuehniella* eggs or *P. citri* mealybugs developed successfully with survival rates above 90% (Table 7). Developmental time ($t=10.035$, $df=25.887$, $P<0.001$ for males and $t=10.828$, $df=62$, $P<0.001$ for females) and adult body weight ($t=-2.542$, $df=62$, $P=0.014$ for males, $t=-3.038$, $df=62$, $P=0.003$ for females) were affected by diet. Ladybirds fed *E. kuehniella* eggs developed nearly 2 days faster and weighed approximately 10% more than those fed mealybugs. Sex ratios were proportionally divided in both groups ($\chi^2=0.037$, $df=1$, $P=0.848$).

<table>
<thead>
<tr>
<th>Rearing method</th>
<th>N</th>
<th>Immature survival (%)</th>
<th>Developmental time (days)</th>
<th>Adult weight (mg)</th>
<th>Sex ratio (% females)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semi-artificial</td>
<td>95</td>
<td>93.7 ± 2.4a*</td>
<td>22.4 ± 0.1a</td>
<td>10.6 ± 0.1a</td>
<td>50.6 ± 5.3a</td>
</tr>
<tr>
<td>Natural</td>
<td>43</td>
<td>90.7 ± 4.5a</td>
<td>24.6 ± 0.2b</td>
<td>9.7 ± 0.3b</td>
<td>48.7 ± 8.1a</td>
</tr>
</tbody>
</table>

*Mean ± SE within a column followed by the same letter are not significantly different ($P>0.05$; Probit (survival, sex ratio) or Student’s t-test (developmental time and adult weight))*

Although the preoviposition period of females offered synthetic wadding as an oviposition substrate was prolonged by about 8 days ($F=12.615$, $df=2$, 62, $P<0.001$), they produced a similar number of eggs as naturally reared females ($\chi^2=3.605$, $df=2$, $P=0.165$). However, the hatching rate of the eggs
laid in artificial substrates was significantly lower than that of eggs deposited near mealybugs ($\chi^2=18.920$, df=1, $P<0.001$ for Semi-artificial 1 and $\chi^2=21.659$, df=1, $P<0.001$ for Semi-artificial 2)(Table 8). No significant differences were recorded in reproduction parameters between the group that was offered clean synthetic wadding and the group that was offered wadding with imbedded *E. kuehniella* eggs (preoviposition period: $P=0.925$ (Tukey post-hoc test); no. of deposited eggs: $\chi^2=3.605$, df=2, $P=0.165$; egg hatch: $\chi^2=0.148$, df=1, $P=0.700$; longevity: $F=0.477$, df=2, 125, $P=0.622$).

Table 8: Reproduction and female longevity of *C. montrouzieri* reared according to a natural rearing system (food: mealybugs, oviposition substrate: mealybug ovisacs), semi-artificial rearing system 1 (food: *E. kuehniella* eggs, oviposition substrate: polyester wadding) or semi-artificial rearing system 2 (food: *E. kuehniella* eggs, oviposition substrate: polyester wadding with imbedded *E. kuehniella* eggs) (Experiment 1).

<table>
<thead>
<tr>
<th>Rearing method</th>
<th>N</th>
<th>Preoviposition period (days)</th>
<th>No. of deposited eggs (eggs/female)</th>
<th>Egg hatch (%)</th>
<th>Longevity (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural</td>
<td>19</td>
<td>7.5 ± 1.1a*</td>
<td>737.7 ± 111.8a</td>
<td>71.9 ± 1.7a</td>
<td>207.1 ± 20.2a</td>
</tr>
<tr>
<td>Semi-artificial 1</td>
<td>21</td>
<td>15.0 ± 1.6b</td>
<td>746.0 ± 130.4a</td>
<td>60.9 ± 1.8b</td>
<td>223.3 ± 15.8a</td>
</tr>
<tr>
<td>Semi-artificial 2</td>
<td>23</td>
<td>15.7 ± 1.0b</td>
<td>468.1 ± 73.8a</td>
<td>59.8 ± 2.3b</td>
<td>229.6 ± 14.2a</td>
</tr>
</tbody>
</table>

*Mean ± SE within a column followed by the same letter are not significantly different ($P>0.05$; Tukey’s test (preoviposition period, longevity), Mann-Whitney U test (No. of deposited eggs) or Probit (egg hatch)).

The developmental parameters of *C. montrouzieri* fed the diets tested in experiment 2 are presented in Table 9. Larvae developed successfully on all diets (survival > 90%) except on bee pollen alone allowing only 20% of the larvae to reach the 4th larval stage and only 1 male to reach the adult stage. Diet influenced both developmental time ($\chi^2=39.834$, df=2, $P<0.001$ for males and for $\chi^2=38.033$, df=2, $P<0.001$ for females) and adult body weight ($F=5.848$, df=2, 70, $P=0.006$ for males and $F=6.314$, df=2, 68, $P=0.003$ for females). Developmental rate (all $P<0.001$; Mann Whitney test) and female body weight (both $P<0.05$; Tukey post-hoc test) of ladybirds fed *E. kuehniella* eggs or a mixture of *E. kuehniella* eggs and pollen were superior to those of their counterparts offered mealybugs. Body weight of males fed the mixture of *E. kuehniella* eggs and pollen was similar to that of males fed mealybugs ($P=0.182$; Tukey post-hoc test). Except for pollen alone, sex ratios were essentially equal on all diets ($\chi^2=0.015$, df=2, $P=0.993$).
Table 9: Survival, developmental time, adult body weight and sex ratio of *C. montrouzieri* fed different food sources (Experiment 2).

<table>
<thead>
<tr>
<th>Food source</th>
<th>N</th>
<th>Immature survival (%)</th>
<th>Developmental time (days)</th>
<th>Adult weight (mg)</th>
<th>Sex ratio (% females)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. kuehniella</em> eggs</td>
<td>52</td>
<td>92.3 ± 4.1a*</td>
<td>22.6 ± 0.2a</td>
<td>11.1 ± 0.2a</td>
<td>9.9 ± 0.2a</td>
</tr>
<tr>
<td><em>E. kuehniella</em> eggs + pollen</td>
<td>49</td>
<td>91.8 ± 4.3a</td>
<td>22.5 ± 0.2a</td>
<td>10.7 ± 0.2a</td>
<td>9.5 ± 0.2a</td>
</tr>
<tr>
<td><em>P. citri</em> mealybugs</td>
<td>51</td>
<td>92.1 ± 4.1a</td>
<td>24.4 ± 0.2b</td>
<td>9.8 ± 0.3b</td>
<td>8.9 ± 0.2b</td>
</tr>
<tr>
<td>Pollen**</td>
<td>47</td>
<td>2.1 ± 2.1b</td>
<td>-</td>
<td>-</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*Means ± SE within a column followed by the same letter are not significantly different (P>0.05; Probit (survival, sex ratio), Tukey’s test (adult weight), Mann-Whitney U test (developmental time)).

**Only 1 male reached the adult stage.

Preoviposition period was affected by diet ($\chi^2=8.481$, df=2, *P=0.014*) and was over 4 days shorter on mealybugs than on diets containing *E. kuehniella* eggs (Table 10). The diet offered to the ladybirds, however, had no significant effect on the number of eggs deposited by the predator in the synthetic wadding over a 30-day period ($F=0.155$, df=2, 68, *P=0.857*) and egg hatch ($\chi^2=0.017$, df=2, *P=0.992*).

Table 10: Reproduction (over 30 days from the onset of oviposition) of *C. montrouzieri* offered different food sources and synthetic wadding as an oviposition substrate (Experiment 2).

<table>
<thead>
<tr>
<th>Food source</th>
<th>N</th>
<th>Preoviposition period (days)</th>
<th>No. of deposited eggs in 30 days (eggs/female)</th>
<th>Egg hatch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. citri</em> mealybugs</td>
<td>23</td>
<td>10.2 ± 0.8a*</td>
<td>208.1 ± 19.9a</td>
<td>86.1 ± 6.9a</td>
</tr>
<tr>
<td><em>E. kuehniella</em> eggs</td>
<td>24</td>
<td>14.8 ± 1.4b</td>
<td>199.2 ± 17.4a</td>
<td>85.3 ± 9.8a</td>
</tr>
<tr>
<td><em>E. kuehniella</em> eggs + pollen</td>
<td>22</td>
<td>15.3 ± 1.5b</td>
<td>193.7 ± 17.4a</td>
<td>86.7 ± 7.0a</td>
</tr>
</tbody>
</table>

*Means ± SE within a column followed by the same letter are not significantly different (P>0.05; Mann Whitney U test (preoviposition period), Tukey’s test (no. of deposited eggs)), Probit (egg hatch)).

4.4 Discussion

The semi-artificial rearing method based on *E. kuehniella* eggs as a factitious food and synthetic wadding as an artificial oviposition substrate successfully supported development and reproduction of the specialist mealybug predator *C. montrouzieri*. *Ephestia kuehniella* eggs appeared to be an even better food source for the developing larvae of the predator than one of *P. citri* mealybugs: larvae reared on flour moth eggs developed faster and reached a higher adult weight than their
counterparts reared on mealybugs. These findings are consistent with the results of Attia et al. (2011a), suggesting that *E. kuehniella* eggs may constitute a nutritionally superior food for *C. montrouzieri* larvae than its natural prey, mealybugs. Earlier studies showed that a number of other predatory insects generally perform better when reared on *E. kuehniella* eggs than when offered their natural prey, which might be attributed to the well balanced amino acid and fatty acid composition of this factitious prey (Fauvel et al. 1987; Cocuzza et al. 1997; Specty et al. 2003; De Clercq et al. 2005; Berkvens et al. 2008b; De Clercq et al. 2014).

Semi-artificially reared females were not only capable of producing viable eggs but also deposited the same number of eggs as naturally reared females. To our knowledge, this is the first report of *C. montrouzieri* ladybirds reproducing in the absence of mealybugs. Chemical cues produced by their natural prey were always considered to be a necessity to stimulate oviposition. For example, *C. montrouzieri* ladybirds reared on eggs of the flour moth *E. kuehniella* or of the grain moth *S. cerealella* depended on mealybug egg masses to trigger oviposition (Plipijuk et al. 1982; Attia et al. 2011a). Merlin et al. (1996) reported that *C. montrouzieri* females reared on *P. citri* mealybugs and offered cotton wool pellets as an oviposition substrate refrained from ovipositing and withheld their eggs until egg masses were met. Finlay-Doney and Walter (2012a) noted that *C. montrouzieri* females were able to oviposit in the absence of ovisacs as long as mealybugs were offered as food source. In the latter study, the presence of mealybug egg masses had a significant impact on the number of deposited eggs, with significantly fewer eggs overall for females that were not provided with ovisacs but only with mealybug nymphs. Likewise, Attia et al. (2011a) showed that ladybirds reared on *E. kuehniella* eggs and offered ovisacs deposited 30% less eggs compared to females provided with mealybugs and ovisacs. In contrast to abovementioned studies, our results prove that *C. montrouzieri* can effectively be reared in the absence of mealybugs without a loss in fecundity.

The equally good reproductive rates observed in *C. montrouzieri* females reared according to the semi-artificial method as compared to the natural rearing system using mealybugs, can be attributed...
to the use of a nutritionally adequate food source for the larvae and adults of the predator and the presence of an acceptable substrate for egg laying by the adult females. The adequacy of both the factitious food and the artificial substrate for rearing the predator is confirmed by the fact that 15 consecutive generations could be produced using this semi-artificial method and in the complete absence of mealybugs in the rearing environment without evidence of loss in fitness. After 15 generations, female ladybirds had an average developmental time of 23.1 ± 0.2 days and reached an average adult body weight of 11.5 ± 0.2 mg. They laid an average of 313 eggs over a time period of 30 days. These data are comparable with (developmental time and body weight) or even better than (fecundity) those in the first generation of rearing on *E. kuehniella* eggs and synthetic wadding (Tables 9 and 10).

It is unclear why the synthetic wadding used in this study was accepted as oviposition substrate while the cotton wool pellets used by Merlin et al. (1996) were refused. Latter authors asserted that thigmotactic cues are key for triggering the oviposition in *C. montrouzieri*, which stresses the importance of the physical properties of the oviposition substrate. A possible explanation for the acceptance of the polyester wadding used in the present study is that it is more successful in mimicking the filamentous structure of mealybug ovisacs than cotton wool pellets.

The semi-artificial rearing system proposed here does have its limitations. A first drawback is a prolongation in preoviposition period from 7 days for the natural rearing method to 15 days for the semi-artificial method. When the ladybirds were fed mealybugs and provided with synthetic wadding an intermediate preoviposition period of 10 days was found. These findings suggest that the oviposition of *C. montrouzieri* is postponed in the absence of ovisacs and potentially associated chemical cues produced by the mealybugs, but that these cues do not seem required *per se* if a suitable oviposition substrate is available. A significant decrease in egg hatch is the second drawback of the semi-artificial rearing system. When the ladybirds were provided with *E. kuehniella* eggs and
synthetic wadding instead of mealybug ovisacs in the first experiment, egg hatch monitored over the entire lifetime of the female decreased from 72% to 60%.

Imbedding *E. kuehniella* eggs into the oviposition substrate with the objective to accelerate reproduction, proved unsuccessful. Apparently, the availability of food for newly emerged larvae in the oviposition substrate does not trigger oviposition by the females of *C. montrouzieri*.

*Ephestia kuehniella* eggs were found to be an effective factitious food source to sustain the development and reproduction of *C. montrouzieri* and show promise to be implemented in a large scale production of this ladybird. As a downside *E. kuehniella* eggs are relatively expensive (De Clercq et al. 2014). The search for cheaper alternatives is recommended to lower the production cost of this and other insect predators further. Different pollens have been shown to be suitable alternative or supplementary food for the development and reproduction of various predatory arthropods (De Clercq et al. 2005; Berkvens et al. 2008a; Bonte et al. 2010; Vandekerkhove and De Clercq 2010; Nguyen et al. 2013; Vangansbeke et al. 2014). The frozen, moist bee pollen tested in the current study was not an adequate food source for immature *C. montrouzieri* as only 1 individual reached the adult stage, which indicates a lack or shortage of certain nutritional components essential for optimal development of *C. montrouzieri*. However, 60% of the larvae reached the 3rd larval stage when fed pollen alone and no loss in fitness was recorded when the flour moth eggs were mixed with pollen. Dissected ladybird larvae fed the mixture of *E. kuehniella* eggs and pollen had high amounts of pollen in their gut, indicating that the larvae not only actively fed on the lepidopteran eggs but also on the pollen. Therefore, mixing *E. kuehniella* eggs with pollen may contribute to an increase in cost-effectiveness of mass production by reducing the input of the nutritionally superior but highly expensive flour moth eggs (De Clercq et al. 2005; Berkvens et al. 2008a; Bonte et al. 2010; Lundgren and Weber 2010; Pilorget et al. 2010; Lundgren et al. 2011; Weber and Lundgren 2011).

In conclusion, *C. montrouzieri* can be successfully reared on *E. kuehniella* eggs as a food source and synthetic wadding as an oviposition substrate. The major drawback of the semi-artificial rearing
method is a delay in the onset of oviposition by about 8 days. However, when the shorter developmental time of larvae fed *E. kuehniella* eggs is taken into consideration (2 days), the semi-artificial rearing system yields a delay of only about 6 days compared to the traditional rearing method. Besides, the semi-artificial rearing method has an important advantage: *C. montrouzieri* can be reared without the need to maintain cultures of mealybugs and potato sprouts or other plant materials. This should make the mass rearing of *C. montrouzieri* less time-consuming, less labour-intensive and more cost-effective. A possible next step in rationalizing the rearing system for this ladybird is the development of an artificial diet (Riddick and Chen 2014). Chumakova (1962) described an artificial diet based on casein and amino acids for *C. montrouzieri* but larvae suffered from high mortality (Hodek 1967). Recent advances in the development of artificial diets for predatory arthropods (Morales-Ramos et al. 2014a) may be helpful in designing an effective artificial diet for this ladybird as well.
Chapter 5

Cold tolerance of Cryptolaemus montrouzieri

Redrafted after:

Maes S, Grégoire JC, De Clercq P Cold tolerance of the predatory ladybird Cryptolaemus montrouzieri. BioControl (under review)
5.1 Introduction

Since 1992 the Australian ladybird *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) is being released in northwestern Europe as an augmentative BCA under protected cultivation and interior landscaping (Malais and Ravensberg 2002). Meanwhile, the ladybird has been reported outside greenhouses in natural environments in Sweden, Norway, Britain and Belgium (Ehnström and Lundberg 1997; Constantine and Majerus 1994; Hansen and Sagvolden 2007), but there is no unequivocal evidence of permanent establishment in those countries. Because *C. montrouzieri* is a (sub)tropical species, it is generally believed not to be sufficiently cold hardy to survive winters in cold and temperate climates, but observations from Britain indicate that the ladybird may survive shorter periods with frost (Halstead 1999). A non-indigenous BCA characterized by high cold tolerance may establish in a new area and cause undesired side-effects on local biodiversity (van Lenteren et al. 2003).

Knowledge of the cold tolerance of *C. montrouzieri*’s may contribute its ERA. The methodology developed by Hart et al. (2002a) to assess cold tolerance of arthropod BCAs builds on several laboratory parameters, including the supercooling point (SCP) and lethal time (LTime) at 5°C. The relationship between these parameters and the chances of survival in the field during winter appears sufficiently robust for an assessment to be based on laboratory studies alone (Hatherly et al. 2005; Boivin et al. 2006; Bale 2011a). The influence of low temperature acclimation and diet on the supercooling ability and lethal time of *C. montrouzieri* was assessed. In the laboratory *C. montrouzieri* is usually cultured under continuous summer conditions, whereas insects in the field are confronted with temperatures that vary with the season (Block 1990; Danks 2005). Because field insects may become acclimatized to colder conditions as temperatures gradually drop in autumn, exposing laboratory cultured *C. montrouzieri* directly to low temperatures may lead to inaccurate predictions of their cold tolerance. Further, diets used in commercial insectaries may have a strong impact on the physiological responses of the natural enemies produced (Grenier and De Clercq 2003) and may thus
also influence their responses to climatic conditions. Therefore, the cold tolerance of *C. montrouzieri* adults offered the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) was compared to those reared on a factitious food source, eggs of the flour moth *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae).

### 5.2 Materials and Methods

#### 5.2.1 Insect cultures

Two laboratory colonies of *C. montrouzieri* were established in 2010 with larvae acquired from Katz Biotech AG (Baruth, Germany) and maintained in a climatic chamber set at 25±1°C, a relative humidity (RH) of 75±5% and a 16:8(L:D)h photoperiod. The first colony was maintained on frozen eggs of the flour moth *E. kuehniella*. Water was provided by way of a moist piece of cotton wadding fitted into a 1.5cm (Ø) plastic dish. A larger piece of dry cotton wadding (5x5cm) was offered to adult beetles and served as an artificial oviposition substrate for females; no mealybugs were used in this rearing system (Chapter 4). The second colony of the predator was reared on the citrus mealybug *P. citri* as described in section 4.2.1.

#### 5.2.2 Experimental set-up

The effect of acclimation on the cold tolerance of *C. montrouzieri* was evaluated by examining the SCP and LTime at 5°C of ladybirds undergoing one of 3 climatic regimes (Table 11). Larvae subjected to climatic regimes 1 and 2 were reared from first instar to adulthood in an incubator set at a temperature of 25±1°C, 75±5% RH and a 16:8(L:D)h photoperiod, whereas larvae of treatment group 3 were exposed to autumn conditions and maintained in an incubator kept at 18±1°C, 75±5% RH and a 8:16(L:D)h photoperiod. Newly emerged larvae (<24h) were taken out of the stock colony reared on *E. kuehniella* eggs (generation 10), placed in polystyrene Petri dishes (Ø: 9cm, height: 1.3 cm) at a
density of 1 (SCP) or 10 (LTime) individuals per Petri dish and transferred to their respective climatic regime. After they reached adulthood, ladybirds undergoing climatic regime 1 were maintained at 25°C for a further 7 days. In contrast, individuals subjected to treatments 2 and 3 were allowed to acclimatize to lower temperatures by keeping them in an incubator set at 10±1°C and a 12:12(L:D)h photoperiod for 7 days (humidity was not controlled during acclimation). Larvae and adults were supplied with *E. kuehniella* eggs and water.

The effect of food source (during larval development and early adult stage) on the cold tolerance of *C. montrouzieri* was assessed by measuring the SCP and LTime at 5°C of 2 populations (Table 11). The first *C. montrouzieri* population (Food source 1) was reared on *E. kuehniella* eggs for 12 generations, whereas the second population (Food source 2) was reared on mealybugs for 8 generations. Newly emerged larvae (<24h) were taken out of their respective stock colony and placed individually in polystyrene Petri dishes at a density of 1 (SCP) or 10 (LTime) individuals per Petri dish. The ladybirds were provided with their respective diet and water throughout the experiments and were maintained at 25±1°C, 75±5% RH and a 16:8(L:D)h photoperiod. Before the cold tolerance of the adult beetles was assessed, they were allowed to acclimate to lower temperatures in an incubator set at 10±1°C and a 12:12(L:D)h photoperiod for 7 days, during which they were also fed.

**Table 11**: Overview of the different experimental treatments to assess the influence of climate and food source on the cold tolerance of *C. montrouzieri*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rearing conditions during development</th>
<th>Adults exposed to the acclimation regime ?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Food source</td>
<td>Temperature</td>
</tr>
<tr>
<td><strong>Climate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Climatic regime 1</td>
<td><em>E. kuehniella</em> eggs</td>
<td>25°C</td>
</tr>
<tr>
<td>Climatic regime 2</td>
<td><em>E. kuehniella</em> eggs</td>
<td>25°C</td>
</tr>
<tr>
<td>Climatic regime 3</td>
<td><em>E. kuehniella</em> eggs</td>
<td>18°C</td>
</tr>
<tr>
<td><strong>Food source</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food source 1</td>
<td><em>E. kuehniella</em> eggs</td>
<td>25°C</td>
</tr>
<tr>
<td>Food source 2</td>
<td><em>P. citri</em> mealybugs</td>
<td>25°C</td>
</tr>
</tbody>
</table>

* acclimation regime: 7 days at 10±1°C and 12:12(L:D)h, using the same diet as during development
** maintained at rearing conditions for 7 days
5.2.3 Measurement of supercooling point

For each diet and acclimation regime, 50 7-day old adult *C. montrouzieri* (25 males and 25 females) were subjected to the supercooling experiment, except for ladybirds reared on mealybugs where only 40 individuals (20 males and 20 females) were tested. The SCP was measured using a Picotech TC-08 thermocouple datalogger and a low temperature programmable Haake Phoenix II CP30 alcohol bath. Each thermocouple was led individually through the lid of a 1.5 ml Eppendorf tube. The insects were attached to the thermocouples with petroleum jelly and the Eppendorf tubes were sealed with Pritt Poster Buddy. The Eppendorf tubes were placed individually in glass tubes which were immersed in the alcohol bath (Berkvens et al. 2012) The starting temperature was set at 25°C (rearing temperature) or 10°C (acclimation temperature). The insects were cooled at 0.5°C/min until the thermocouple registered the release of exothermal heat, at which point the supercooling temperature is reached. Insects were weighed upon adult emergence and before being subjected to the experiments, using a semi-microbalance Sartorius Genius ME215P (Sartorius AG, Goettingen, Germany)(±0.01mg). This was done in order to check whether supercooling ability was correlated to body weight and weight loss or gain between adult emergence and the time of SCP measurement.

5.2.4 Measurement of lethal time

For each diet and acclimation regime, 60 Petri dishes each containing 10 adult *C. montrouzieri* (5 males and 5 females) were set up at room temperature. Food and moisture were provided *ad libitum* throughout the experiment. Predators from treatment groups at 25°C and 18°C were subsequently transferred to incubators set at 15°C and 10°C, and held there for 30min each time to avoid possible mortality due to cold shock, before being finally transferred to an incubator set at 5°C±1°C. Individuals undergoing the acclimation treatment at 10°C for 7 days were directly transferred to 5°C. Throughout exposure to 5°C, the insects were kept in total darkness and relative humidity was not controlled. Three Petri dishes (in total 30 individuals) were taken from the incubator set at 5°C at
regular time intervals, and transferred subsequently to incubators set at 10°C and 15°C, where they were held for 30 min each time. The insects were then transferred to 25°C and kept there for 24 h, after which survival was monitored.

5.2.5 Statistical analysis

All data were analyzed using SPSS 21.0 (SPSS Inc. 2009). For the supercooling experiment, immature survival rates were considered as binary data, which enabled us to calculate standard errors on percentages. The means were compared by means of a logistic regression. This regression is a generalized linear model using a probit (log odds) link and a binomial error function (McCullagh and Nelder 1989). P-values below 0.05 were considered significant. Developmental times, body weights, and SCP-values of ladybirds undergoing different climatic regimes were analyzed using a one-way analysis of variance (ANOVA). Means were separated using Tukey or Tamhane post hoc tests when a Levene test indicated homoscedasticity or heteroscedasticity, respectively. The developmental parameters, SCP-values and weight loss during acclimation of ladybirds receiving different food sources were compared using Student’s t-tests. The relationship between SCP on the one hand and sex, developmental time, adult body weight, weight loss or gain between adult emergence and the time of SCP measurement, and body weight prior to testing on the other hand, was assessed with a Pearson’s correlation test (SPSS Inc. 2009).

The results from the lethal time experiments were analyzed using Probit analysis in order to estimate the time required to kill 10, 50 and 90% of the population at a temperature of 5°C. Significant differences were identified by non-overlapping fiducial limits (Hart et al. 2002a).
5.3 Results

The supercooling ability of *C. montrouzieri* was affected by the climatic regimes the ladybird was exposed to before testing (F=156.72, df=2, 148, P<0.001)(Table 12). Ladybirds reared under climatic regime 2 and 3 during their immature stages had an average SCP which was 6.9°C and 7.5°C lower, respectively, than that of their counterparts maintained under climatic regime 1 (both P<0.001; Tamhane test). There was no significant difference in SCP between individuals exposed to climatic regime 2 and 3 (P=0.377; Tamhane test). The food offered to *C. montrouzieri* during its immature development and early adult stage had a significant effect on its supercooling ability (t=3.99, df= 87, P<0.001): the SCP of ladybirds fed food source 2 was 1.6°C higher than that of ladybirds provided with food source 1. None of the tested adult ladybirds survived the freezing treatment.

**Table 12**: Survival, developmental time, adult body weight and SCP of *C. montrouzieri* ladybirds reared under different climatic regimes and fed different diets.

<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>N</th>
<th>Survival (%)</th>
<th>Developmental time (days)</th>
<th>Adult body weight (mg)</th>
<th>SCP (°C)</th>
<th>Range of SCP (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Climate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Climatic regime 1</td>
<td>50</td>
<td>94.3 ± 3.2a</td>
<td>23.4 ± 0.2a</td>
<td>11.1 ± 0.2a</td>
<td>-9.9 ± 0.4a</td>
<td>-5.6 to -16.2</td>
</tr>
<tr>
<td>Climatic regime 2</td>
<td>48</td>
<td>92.3 ± 3.7a</td>
<td>23.2 ± 0.2a</td>
<td>11.0 ± 0.2a</td>
<td>-16.8 ± 0.5b</td>
<td>-11.6 to -19.6</td>
</tr>
<tr>
<td>Climatic regime 3</td>
<td>51</td>
<td>79.7 ± 5.0b</td>
<td>54.2 ± 0.2b</td>
<td>8.8 ± 0.3b</td>
<td>-17.4 ± 0.2b</td>
<td>-12.0 to -20.7</td>
</tr>
<tr>
<td><strong>Food source</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food source 1</td>
<td>50</td>
<td>90.9 ± 3.9a</td>
<td>23.7 ± 0.2a</td>
<td>10.8 ± 0.2a</td>
<td>-17.2 ± 0.2b</td>
<td>-13.1 to -20.4</td>
</tr>
<tr>
<td>Food source 2</td>
<td>39</td>
<td>92.9 ± 4.0a</td>
<td>24.4 ± 0.1b</td>
<td>10.0 ± 0.2b</td>
<td>-15.6 ± 0.3a</td>
<td>-11.7 to -20.3</td>
</tr>
</tbody>
</table>

*Means ± SE within a column followed by the same letter are not significantly different (P>0.05; Climatic regime: Probit (Wald-Chi²)(survival), Tukey test (developmental time), Tamhane test (body weight, SCP), Food source: Probit (Wald-Chi²)(survival), t-test (developmental time, body weight, SCP))*

Immature survival was affected by temperature and photoperiod during development (χ²=6.80, df=2, P=0.033): the survival rate of larvae and pupae reared under climatic treatment 3 was approximately 13% lower than that of those maintained under climatic regime 2 (both P<0.05). In contrast, the food offered to the larvae had no influence on immature survival (χ²=0.12, df=1, P=0.729). Developmental time was both affected by climatic regime (F=8063.65, df=2, 148, P<0.001) and diet (t=−2.77, df=70.86, P=0.007). Larvae and pupae reared under climatic regime 1 or 2 developed approximately
31 days faster than those maintained under climatic regime 3 (both P<0.001), whereas predators fed food source 2 developed ca. 1 day slower than those given food source 1 (t=-2.77, df=70.86, P=0.007). Climatic regime during development (F=43.60, df=2, 148, P<0.001) and food source (t=3.48, df=87, P=0.001) both influenced adult body weight: ladybirds exposed to climatic treatment 3 weighed approximately 20% less than those exposed to climatic regime 1 or 2 (both P<0.001). Predators reared on diet 1 gained a 7% higher body weight than their counterparts reared on diet 2. The body weight of adults undergoing an acclimation period of 7 days decreased, whereas adults maintained at rearing conditions gained weight (Figure 17). Weight loss in adults during acclimation was more pronounced in ladybirds reared at 18°C than in those reared at 25°C (F=128.94, df=2, 148, P=0.008), and in ladybirds fed E. kuehniella eggs versus those fed mealybugs (t=4.96, df=87, P<0.001).

No significant correlations could be found between SCP and sex (r=0.104, P=0.372, n=149 for climate; r=-0.119, P=0.269, n=89 for food source), SCP and adult body weight (r=-0.237, P=0.224, n=149 for climate; r=0.163, P=0.127, n=89 for food source), SCP and weight loss during acclimation (r=0.035, P=0.730, n=99 for climate; r=0.094, P=0.380, n=89 for food source), SCP and weight gain for non-acclimated ladybirds (r=0.335, P=0.121, n=50) and SCP and body weight just before testing (r=-0.188, P=0.107, n=149 for climate; r=0.139, P=0.193, n=89 for food source).

The lower lethal times for 10, 50 and 90% mortality (LTime\textsubscript{10,50,90}) at a temperature of 5°C for C. montrouzieri reared under different climatic regimes and with different food sources are presented in Table 13. Based on overlapping fiducial limits, neither climate nor diet had a significant effect on the lethal time of C. montrouzieri. Overall, the time required to kill 50% of the population ranged from 12.8 to 14.4 days. All individuals died by day 24.
**Figure 17**: Weight loss or gain between adult emergence and time of SCP measurement (A) and body weight before SCP measurement (B) of *C. montrouzieri* ladybirds reared under different climatic regimes (climatic regime 1, 2 and 3 in white, black and grey, respectively) and fed different food sources (diet 1 and 2 in white and black, respectively). Within each factor, graph bars (means ± SE) with the same letter are not significantly different (P>0.05; Climate: Tamhane tests, Food: t-tests).

**Table 13**: Lethal time _LT_{10,50,90} [±95% fiducial limits] at 5°C for *C. montrouzieri* ladybirds reared under different climatic regimes and fed different diets.

<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>LT_{10} (days)</th>
<th>LT_{50} (days)</th>
<th>LT_{90} (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Climate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Climatic regime 1</td>
<td>9.3 [8.3-10.2]</td>
<td>13.3 [12.5-14.1]</td>
<td>17.3 [16.4-18.3]</td>
</tr>
<tr>
<td><strong>Food source</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.4 Discussion

Low temperature acclimation had a positive effect on the freezing tolerance of C. montrouzieri. Ladybirds reared under continuous summer conditions (25°C, long days) froze at a temperature which was approximately 7°C higher than the freezing temperature of ladybirds undergoing a short acclimation period at 10°C before testing. Within acclimated ladybirds, no significant difference in SCP was observed between ladybirds exposed to climatic regime 2 or climatic regime 3. This finding suggests that exposing C. montrouzieri taken directly from the laboratory culture to a short acclimation period is sufficient to detect a possible acclimation response. In chapter 8 is shown that a similarly short acclimation period had a significant impact on the SCP of the predatory bug M. pygmaeus: bugs acclimated to lower temperatures froze at a lower temperature than their counterparts maintained at rearing conditions.

An increase in cold tolerance after acclimation might indicate an insect’s potential to gradually adjust to decreasing temperatures. Although cold acclimation had a positive effect on the supercooling ability of C. montrouzieri, no acclimation response could be detected in terms of its lethal time. This is in contrast with earlier studies which either demonstrated an acclimation response for both SCP and lethal time or an acclimation effect for lethal time but not for SCP. For example, both SCP and lethal time measurements indicated that the parasitoid Spathius agrili Yang (Hymenoptera: Braconidae) was less tolerant to low temperatures when reared under warm temperature and long photoperiod conditions than when reared under cold temperature and short photoperiod (Hanson et al. 2013). Further, acclimated individuals of the predatory bug Macrolophus caliginosus Wagner (Hemiptera: Miridae), the parasitoid Eretmocerus eremicus Rose and Zolnerowich (Hymenoptera: Aphelinidae) and the predatory mites Neoseiulus californicus (McGregor) and Typhlodromips montdorensis (Schicha)(Acari: Phytoseiidae) were more cold hardy than non-acclimated individuals based on their lethal time in exposures at -5°C, 0°C or 5°C, whereas acclimation had no effect on their supercooling abilities (Hart et al. 2002a, b; Hatherly et al. 2004; Tullett et al. 2004).
The food source offered to *C. montrouzieri* during its immature development and early adult stage affected its supercooling ability. Predators reared on *E. kuehniella* eggs had a lower SCP than their counterparts offered *P. citri* mealybugs. Likewise, *M. pygmaeus* bugs reared on *E. kuehniella* eggs were found to be more cold tolerant than those fed an artificial diet based on egg yolk (Chapter 8). Specty et al. (2003) reported that *E. kuehniella* eggs are rich in fatty acids and amino acids and pointed out that these nutrients may protect a predator fed on this factitious food against extreme temperatures by delivering components that promote winter survival. As SCP measurements evaluate an insect's resistance to a brief cold exposure, whereas lethal time measurements assess its cold hardiness when faced with a long-term cold exposure (Chown and Terblanche 2006), a more pronounced effect of the lepidopteran eggs with their higher caloric value could be expected on lethal time than on SCP. However, no significant difference in lethal times between ladybirds fed *E. kuehniella* eggs and those offered *P. citri* mealybugs could be detected.

Based on our experiments and information in the literature, it is deemed unlikely that *C. montrouzieri* could establish outdoors in northwestern Europe. Optimal temperatures for development and reproduction of this ladybird are 25°C and above (Fisher 1963; Babu and Azam 1987). In the present study, exposing the immatures to a temperature of 18°C and short day conditions led to a drastic increase in developmental time (from 23 days to 54 days) and a substantial decrease in both immature survival (13%) and adult body weight (20%). Iperti (1999) showed that *C. montrouzieri* does not possess any diapause trait but resists drastic changes in climate by reducing its speed of development. The predator's developmental threshold is relatively high and estimated at 14°C (Fisher 1963; Malais and Ravensberg 2002). Further, our experiments showed that *C. montrouzieri* did not survive temperatures below its supercooling point and can therefore be classified as freeze intolerant (Sømme 1982). Besides, all *C. montrouzieri* adults had died by day 24 when exposed to 5°C, indicating its susceptibility to chilling injury due to above-zero cold temperatures. Hatherley et al. (2005) reported a strong positive correlation between maximum field survival and survival at 5°C.
in the laboratory for several arthropod BCAs and this trend has been confirmed by subsequent studies (Hatherly et al. 2008; Hughes et al. 2009). When applying the relationship between LTime$_{50}$ at 5°C and field survival calculated by Hatherley et al. (2005) to our dataset, it can be predicted that *C. montrouzieri* would not persist longer than 50 days in the field in western European winters. Thus, the above data suggest that *C. montrouzieri* is unlikely to permanently establish in the cooler temperate climate of western Europe.
Chapter 6

Dispersal potential of predatory ladybirds as measured by a computer-monitored flight mill

Redrafted after:

6.1 Introduction

Information on their dispersal capacity is of crucial importance for the ERA of predators and parasitoids with oligophagous or polyphagous feeding habits that will be released in large numbers. A candidate BCA with these characteristics could have a negative impact on non-target fauna when dispersal from the release site is extensive (Bale 2011a, b). However, there is still no standard protocol available to predict the dispersal capacity of natural enemies as candidate BCAs. In this chapter, the usefulness of the flight mill apparatus as a tool in a risk assessment procedure for predatory ladybirds was investigated. This apparatus has mostly been used as a convenient and relatively inexpensive way to assess the migratory performance of insects (Riley et al. 1997; Nedved et al. 2001), to understand the ecological consequences of flight (Bruzzone et al. 2009) and to investigate the effect of flight performance on an insect’s physiological state (Luo et al. 2002; Amat et al. 2012).

We compared the flight performance of three species of predatory ladybirds in a computer-monitored flight mill. The first species selected, the two-spotted ladybird Adalia bipunctata (L.) (Coleoptera: Coccinellidae), is native to Europe. The second and third species selected are not indigenous to Europe: the invasive, harlequin ladybird Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae) and the Australian ladybird Cryptolaemus montrouzieri Mulsant (Coleoptera: Coccinellidae). In addition, the effect of diet on the flight potential of these ladybirds was assessed. Therefore, we compared the flight potential of ladybirds fed on their natural prey with that of their counterparts reared on a factitious food source (frozen eggs of the Mediterranean flour moth Ephesia kuehniella Zeller (Lepidoptera: Pyralidae)).
6.2 Materials and Methods

6.2.1 Insect cultures

A laboratory colony of *H. axyridis* was initiated in 2011 by collecting individuals from an established wild population in a park in Ghent (Belgium). The ladybirds were reared on frozen eggs of *E. kuehniella* as described by De Clercq et al. (2003) and Berkvens et al. (2008a). A second *H. axyridis* colony was established by taking insects from the stock colony (generation 15) and feeding them on the pea aphid *Acyrthosiphon pisum* (Harris)(Hemiptera: Aphididae) instead of *E. kuehniella* eggs. Both *H. axyridis* populations were maintained at 23±1°C, 65±5% relative humidity (RH) and a 16:8h (L:D) photoperiod. Ladybirds reared for 17 generations on *E. kuehniella* eggs and for 2 generations on *A. pisum* were subjected to the experiments.

A first laboratory colony of *C. montrouzieri* was reared on *E. kuehniella* eggs as described in section 5.2.1. A second colony was maintained on *P. citri* mealybugs as described in section 4.2.1. Experiments were done using individuals of the 5th generation of both populations.

Two populations of *A. bipunctata* were initiated from specimens supplied by CRA-W (Gembloux, Belgium) in 2012: one population was fed a mixture of frozen *E. kuehniella* eggs and bee pollen, as described by De Clercq et al. (2005), while a second population was reared on *A. pisum* aphids. Both populations were kept in incubators set at 23±1°C, 65±5% RH and a 16:8(L:D)h photoperiod. Insects of the second generation were used in the experiments.

6.2.2 Experimental set-up

To investigate whether the flight activity of ladybirds was influenced by food source or gender, 120 newly emerged males and females of each species (*H. axyridis*, *C. montrouzieri* and *A. bipunctata*) and each population (naturally vs. artificially reared) were paired and caged in small Petri dishes. Adults of the different populations were allowed to feed on their respective diet for 7 to 10 days...
before being subjected to the experiments. Morph type was determined for *H. axyridis* (f. *succinea* (referred to as non-melanic *H. axyridis* individuals), f. *conspicua* (melanic *H. axyridis* individuals)) and *A. bipunctata* (f. *typica* (non-melanic *A. bipunctata* individuals), f. *sublunata* (melanic *A. bipunctata* individuals)) (Majerus and Kearns 1989; Osawa and Nishida 1992). In order to check whether the flight parameters were correlated with body weight, insects were weighed using a semi-microbalance Sartorius Genius ME215P (Sartorius AG, Goettingen, Germany) (±0.01mg) before being attached to the flight mills.

All flight mill trials were performed in the laboratory facilities at LUBIES (ULB, Brussels) in an air conditioned room where temperature remained constant (23°C) throughout the experiments. The 10 flight mills were placed in Kewlox© cabinets (Figure 18). Each compartment (50*50*40cm) was illuminated by a Fluorescent 10W tube fixed at the ceiling. To keep the relative humidity in the cabinets around 60% an electric air humidifier (Vicks©, V-5200) was installed in the testing room and a Petri dish covered with wet filter paper was placed in each compartment (Figure 18).

*Figure 18:* Overview of the ten Kewlox© cabinets (left). Each cabinet contains one flight mill (right).
The base of each flight mill consisted of a crystal polystyrene box (Ø: 4.8 cm, height: 2 cm) filled with sand to increase stability (Figure 19). A syringe needle (Terumo©) glued to the centre of the box acted as a stator. A steel arm (Ø: 0.3 mm) bent at both ends and transversely inserted into the needle operated as the rotor (length: 8 cm). Insects were secured to the rotor arm by a small amount of Pritt Poster Buddy fixed to their pronotum. An infrared beam emitted by a photogate was interrupted by a black opaque label attached to the rotor arm to record the time elapsed during each rotation. In each compartment, a signal transmitter was positioned on the wooden ground surface while the receiver, attached to a metal frame, was located 10 cm above the transmitter. The receptor cells were connected to a data acquisition board (National Instruments, NI USB 6501, 6.5mA) and the program UlbDaqNiMoulin (Authors: T. Ravet and A. Jannin) registered the transit time of the rotations.

![Figure 19](image.png)

**Figure 19:** Schematic overview of the flight mill used in the experiments (A: flight mill base, B: stator, C: rotor, D: ladybird, E: photogate, F: black flag, G: signal receiver, H: metal frame).

### 6.2.3 Evaluation of flight potential

Because a single flight parameter might produce misleading results or fail to reveal important flight components (Dingle 1985; Luo et al. 2002), several flight parameters were analyzed to compare the flight potential of *H. axyridis*, *C. montrouzieri* and *A. bipunctata*. So, for each trial flight distance (in km), flight duration (in min), number of breaks (a break being defined as no passage for more than 5
seconds), average flight velocity (in m/s; total distance divided by total time) and maximum flight velocity (in m/s) were recorded. Each individual was attached to a flight mill for a 1 hour time period and was used only once.

6.2.4 Statistical analysis

The flight parameters (flight distance, flight duration, number of breaks, average flight velocity and maximum flight velocity) were analyzed using a three-way analysis of variance (ANOVA) with following factors: species, food source and gender. The means were separated using Tamhane tests because a Levene test indicated heteroscedasticity. When a significant two-fold interaction between species and food source was detected, the data were analyzed for the interacting factors separately and means were subsequently separated using Tamhane tests. The flight parameters of the different morphotypes of *H. axyridis* and *A. bipunctata* were compared using Student’s heteroscedastic t-tests as the Levene test indicated unequal variances. P-values below 0.05 were considered significant. The relationship between body weight and the flight parameters was assessed with a Pearson’s correlation test. All data were analyzed using SPSS 21.0 (SPSS Inc. 2009).

6.3 Results

A three-way analysis of variance (ANOVA) showed no three-factorial interactions between the factors species, diet and gender for the parameters flight distance (P=0.868), flight duration (P=0.867), number of breaks (P=0.809), average velocity (P=0.747) and maximum velocity (P=0.054)(Table 14). Further, no two-fold interactions between species and gender, and food and gender were observed. The two-fold interactions between species and food source, however, were significant for all flight parameters tested, except for the number of breaks (P=0.066).
Table 14: Three-way ANOVA results indicating the effect of species, food source and gender on the flight parameters of *H. axyridis*, *C. montrouzieri* and *A. bipunctata*.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Flight distance (F)</th>
<th>Flight duration (F)</th>
<th>No. of breaks (F)</th>
<th>Average velocity (F)</th>
<th>Maximum velocity (F)</th>
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<tr>
<td>Species</td>
<td>172.778</td>
<td>109.265</td>
<td>17.544</td>
<td>42.499</td>
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<td></td>
<td>&lt;0.001</td>
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<td>Food</td>
<td>93.423</td>
<td>5.247</td>
<td>0.848</td>
<td>74.201</td>
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<td>0.487</td>
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<td>Gender</td>
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<td>0.033</td>
<td>1.288</td>
<td>6.554</td>
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<tr>
<td></td>
<td>0.310</td>
<td>0.172</td>
<td>0.857</td>
<td>0.275</td>
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<tr>
<td>Species x food</td>
<td>32.032</td>
<td>9.725</td>
<td>2.738</td>
<td>17.877</td>
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<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.066</td>
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<td>&lt;0.001</td>
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<td>Species x gender</td>
<td>0.024</td>
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<tr>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.066</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Food x gender</td>
<td>0.976</td>
<td>0.841</td>
<td>0.937</td>
<td>0.309</td>
<td>0.889</td>
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<td></td>
<td>0.954</td>
<td>0.769</td>
<td>0.620</td>
<td>0.866</td>
<td>0.984</td>
</tr>
<tr>
<td>Species x food x gender</td>
<td>0.141</td>
<td>0.143</td>
<td>0.213</td>
<td>0.292</td>
<td>2.947</td>
</tr>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.868</td>
<td>0.867</td>
<td>0.809</td>
<td>0.747</td>
<td>0.054</td>
</tr>
<tr>
<td>Error</td>
<td>452</td>
<td>452</td>
<td>452</td>
<td>452</td>
<td>452</td>
</tr>
</tbody>
</table>

Because gender had no influence on the parameters flight distance, flight duration and average velocity (all P>0.172) and because the interaction between species and food source was significant for these parameters (all P<0.001), data of males and females were pooled and subsequently analyzed for food and species separately (Figure 20). ANOVA showed significant differences for flight distance (F=155.86, df=5, 463, P<0.001), flight duration (F=90.79, df=5, 433, P<0.001) and average flight speed (F=37.75, df=5, 432, P<0.001). *Harmonia axyridis* outperformed *A. bipunctata* in total distance flown irrespective of gender and food source (all P<0.001). *Cryptolaemus montrouzieri* ladybirds showed an intermediate flight distance between *H. axyridis* and *A. bipunctata*.

When *C. montrouzieri* was reared on *E. kuehniella* eggs its total distance flown matched that of *H. axyridis* fed on *A. pisum* (P=0.916). On the other hand, when *C. montrouzieri* was offered *P. citri* mealybugs its flight distance was similar to that of *A. bipunctata* fed *E. kuehniella* eggs (P=0.212). The total distance flown of *H. axyridis* and *C. montrouzieri* fed *E. kuehniella* eggs exceeded that of the ladybirds fed their natural prey (both P<0.003).
Harmonia axyridis had a greater flight duration than C. montrouzieri and A. bipunctata irrespective of food source (all P<0.001). The flight duration of C. montrouzieri fed E. kuehniella eggs matched that of A. bipunctata fed aphids, whereas C. montrouzieri reared on its natural prey spent the same time flying as A. bipunctata provided with E. kuehniella eggs (both P>0.955).

Harmonia axyridis fed E. kuehniella eggs outranked all C. montrouzieri and A. bipunctata populations in terms of flight velocity (all P<0.001). Adalia bipunctata females maintained on A. pisum flew 5.5 times slower than H. axyridis females given E. kuehniella eggs. When the ladybirds were reared on their natural food sources, C. montrouzieri had a higher average flight speed than H. axyridis and A. bipunctata (P<0.002).

The two-fold interaction between species and food source was also significant for the parameter maximum flight speed (P<0.001). In contrast to former flight parameters, gender affected maximum velocity (P<0.001), with female ladybirds reaching a higher maximum flight speed than males (Table 14). Data were analyzed for the factors species, food and gender separately (Figure 21). ANOVA indicated significant differences (F=37.11, df=11, 456, P<0.001). Harmonia axyridis males and females fed E. kuehniella eggs flew significantly faster than their counterparts reared on aphids and than C. montrouzieri or A. bipunctata fed natural or artificial food (all P<0.003).
Dispersal potential of *C. montrouzieri*

**Figure 20:** Flight distance (A), flight duration (B) and average flight speed (C) of *H. axyridis*, *C. montrouzieri* and *A. bipunctata* fed on factitious (grey bar) or natural prey (white bar). Data of males and females were pooled as factor analysis showed no influence of gender. Graph bars (means ± SE) with the same letter are not significantly different (P>0.05; Tamhane test).
For the parameter number of breaks no two-fold interactions were detected (all $P>0.066$). In contrast to gender and food source, species was found to have an impact on the number of breaks ($P<0.001$)(Table 14). ANOVA showed significant differences ($F=42.08$, df=2, 432, $P<0.001$). *Harmoniaaxyridis* ($5.8 \pm 0.5$ breaks, mean $\pm$ SE) flew more frequently than *Cryptolaemusmontrouzieri* ($17.8 \pm 1.6$ breaks) and *A. bipunctata* ($29.2 \pm 2.9$ breaks)(both $P<0.001$). *Cryptolaemusmontrouzieri* was a more frequent flyer than *A. bipunctata* ($P=0.002$).

The adult body weight of *H. axyridis* and *C. montrouzieri* was positively correlated with flight distance (*H. axyridis*: $n=156$, $r=0.278$, $P<0.001$, *C. montrouzieri*: $n=188$, $r=0.210$, $P=0.004$) and maximum flight speed (*H. axyridis*: $n=156$, $r=0.345$, $P<0.001$, *C. montrouzieri*: $n=188$, $r=0.163$, $P=0.030$) and negatively correlated with flight duration (*H. axyridis*: $n=156$, $r=-0.224$, $P=0.005$, *C. montrouzieri*: $n=188$, $r=-0.151$, $P=0.045$). A strong correlation was also found between body weight and average flight speed ($n=156$, $r=0.287$, $P<0.001$) in *H. axyridis*. In contrast, no correlations between body weight and the flight parameters were detected for *A. bipunctata* (flight distance: $n=120$, $r=-0.079$, $P=0.391$; number of breaks: $n=120$, $r=0.032$, $P=0.727$; flight duration: $n=120$, $r=0.021$, $P=0.834$; average flight velocity: $n=120$, $r=-0.129$, $P=0.160$; maximum flight velocity: $n=120$, $r=-0.113$, $P=0.219$).
Differences between the flight performances of the morphotypes of *H. axyridis* and *A. bipunctata* were detected (Table 15). The red (or non-melanic) morphs of *H. axyridis* flew longer distances (t=3.450, df=154, P=0.001) with a higher average speed (t=3.597, df=154, P<0.001) and were able to reach a higher maximum flight speed (t=7.111, df=153.667, P<0.001) than the black (or melanic) morphs. Morphotype did not influence the flight distance in *A. bipunctata* but had a role in the activity/rest pattern of the ladybirds. Black individuals of the latter species significantly needed more breaks (t=2.094, df=117.972, P=0.038) while red individuals spent longer time in rest (t=2.818, df=119.995, P=0.006).

**Table 15:** Flight parameters of (non-)melanic individuals of *H. axyridis* and *A. bipunctata*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Morphotype</th>
<th>N</th>
<th>Flight distance (km)</th>
<th>Flight duration (min)</th>
<th>No. of breaks (#)</th>
<th>Average velocity (m/s)</th>
<th>Maximum velocity (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. axyridis</em></td>
<td>non-melanic</td>
<td>114</td>
<td>2.6 ± 0.1*</td>
<td>50.3 ± 1.2</td>
<td>6.0 ± 0.5</td>
<td>0.83 ± 0.03</td>
<td>2.07 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>melanic</td>
<td>42</td>
<td>2.0 ± 0.2</td>
<td>47.6 ± 1.98</td>
<td>5.2 ± 0.8</td>
<td>0.68 ± 0.04</td>
<td>1.5 ± 0.04</td>
</tr>
<tr>
<td><em>A. bipunctata</em></td>
<td>non-melanic</td>
<td>27</td>
<td>0.2 ± 0.1</td>
<td>22.1 ± 1.57</td>
<td>18.1 ± 4.8</td>
<td>0.61 ± 0.08</td>
<td>1.08 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>melanic</td>
<td>93</td>
<td>0.4 ± 0.1</td>
<td>11.75 ± 3</td>
<td>33.3 ± 5.4</td>
<td>0.68 ± 0.05</td>
<td>1.08 ± 0.05</td>
</tr>
</tbody>
</table>

*Means ± SE; statistical analysis in text*

### 6.4 Discussion

The aphidophagous coccinellids *H. axyridis* and *A. bipunctata* disperse locally in response to prey densities and make migratory flights to and from their overwintering sites (Hodek and Honěk 1996; Brown et al. 2008a). Because this feature of long-distance migration is generally less developed in coccidophagous species (Hodek and Honěk 1996, Iperti 1999) and because *C. montrouzieri* is the smallest species tested, we expected *H. axyridis* and *A. bipunctata* to achieve better performances in flight mill experiments than *C. montrouzieri*. Further, *H. axyridis* is known to be a powerful flier (Obata 1986; Hodek et al. 1993; Tourniaire et al. 2000), which is believed to be one of the mechanisms underlying its high degree of invasiveness (Brown et al. 2008a, b; van Lenteren et al.
2008; Berkvens et al. 2009; Brown et al. 2011). For the above reasons, it was expected that *H. axyridis* would outcompete the other tested coccinellid species in the flight mills. This hypothesis was only partially confirmed. Indeed, when the ladybirds were reared on *E. kuehniella* eggs, *H. axyridis* flew further than *C. montrouzieri* and *A. bipunctata*, but when they were fed on natural prey, the total distance flown by *C. montrouzieri* was statistically similar to that of *H. axyridis*. *Cryptolaemus montrouzieri* ladybirds fed *E. kuehniella* eggs also flew further than the larger *A. bipunctata* individuals fed the same factitious prey. Cock (2013) discussed the potential non-target impacts associated with the introduction of *C. montrouzieri* for the biological control of the hibiscus mealybug *Maconellicoccus hirsutus* Green in Grenada and also concluded that the dispersal capacity of *C. montrouzieri* should not be underestimated. Despite the less ephemeral nature of their coccidophagous prey, *C. montrouzieri* ladybirds should be classified as high density predators likely to disperse when prey populations are reduced (Cock 2013; De Clercq et al. 2011).

Our results confirm that body size of a species is no reliable indicator to compare the dispersal potential of members of the same taxonomic family. The relatively weak performance of *A. bipunctata* compared to *H. axyridis* matches the observation that the former species is characterized by a shorter distance of most dormancy sites from the breeding habitat (Hemptinne 1989; Hodek and Honĕk 1996). Prior studies revealed a link between dispersal capacity measured in a flight mill system and capacity for long distance migration to overwintering sites observed in the field. Rankin and Rankin (1980) and Nedvĕd et al. (2001) studied the migration behaviour of the convergent ladybird *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) and the seven-spotted ladybird *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), a species known for its long distance migrations and a species with a rather short migratory flight, respectively. While Rankin and Rankin (1980) reported that 60% the individuals of the long distance migrant *H. convergens* flew longer than 30 min, the maximum flight duration observed for the short distance migrant *C. septempunctata* was only 20 min.
For both *H. axyridis* and *C. montrouzieri* a positive correlation between adult body weight, flight distance and velocity, and a negative correlation between body weight and flight duration was discovered. Within each species heavier ladybirds compensated their shorter flight duration by a higher flight speed and flew longer distances than lighter ladybirds. In contrast, no significant correlations between body weight and the flight parameters were detected for *A. bipunctata*. Likewise, positive correlations between adult weight and flight performance have been reported in a number of insect species (Shirae 1995; Fischbein et al. 2011; Bruzone et al. 2009; Kaufmann et al. 2013), whereas these were not found in others (Gu and Barker 1995).

Besides information on the total distance flown, flight mill experiments also reveal information on the rest/activity pattern of insects. In the present study, we found that *C. montrouzieri* and *A. bipunctata* needed more breaks than *H. axyridis*, indicating that the former species may disperse more gradually than *H. axyridis*. *Cryptolaemus montrouzieri* and *A. bipunctata* will forage for prey and oviposition sites within a restricted area. If suitable prey is present in sufficient quantities, the coccinellids will only gradually disperse further (Hodek and Honěk 1996). The capacity to fly great distances (measured here as total flight distance) combined with the ability to fly these distances with a minimum of resting pauses (measured here as the number of breaks) could help explain the rapid spread of *H. axyridis* over the European and North-American continent (Koch et al. 2006; Brown et al. 2008a).

The food source offered to the ladybirds affected their total distance flown, duration of flight and flight velocity, but the interactions between food source and species were also found to be significant. Overall, ladybirds fed *E. kuehniella* eggs flew further, at a higher speed and spent more time flying than ladybirds reared on natural prey. However, these differences were only found to be significant for *H. axyridis*. Wanner et al. (2006) showed that nectar with different nutritional values had a different effect on flight activity in the parasitoid *Cotesia glomerata* (L.)(Hymenoptera: Braconidae). In contrast, Fischbein et al. (2011) reported that the flight parameters of another
parasitoid, *Ibalia leucospoides* Hochenwarch (Hymenoptera: Ibiidae), were not affected by prior access to food, but hypothesized that such an effect may manifest itself on subsequent days of flight. The factitious food source used in our experiments, *E. kuehniella* eggs, was considered to be a better food for *H. axyridis* than pea aphids (Specty et al. 2003; Berkvens et al. 2008b). Specty et al. (2003) found that *E. kuehniella* eggs were nutritionally superior to *A. pisum* in terms of amino acid and fatty acid content and composition, which may be essential nutrients to fuel flight in *H. axyridis*.

No significant influence of gender on the flight parameters of the tested ladybirds was detected except that female ladybirds were able to reach a higher maximum flight speed than males. Further, the influence of morph type on the flight capacity of *H. axyridis* and *A. bipunctata* was not straightforward. While non-melanic *H. axyridis* ladybirds outcompeted the melanic individuals in both flight distance and flight speed, no effect of melanism on these parameters was recorded for *A. bipunctata*. However, the rest/activity pattern of latter species was affected: black individuals took more breaks while red individuals spent more time resting. Although the low frequency of melanic morphs in most *A. bipunctata* populations suggests that they are at a considerable selective disadvantage (Majerus and Kearns 1989), our flight mill output indicates that this disadvantage is not due to lower flight performances. Our experiments also showed a greater flight capacity of red *H. axyridis* morphs. Prior studies have indicated that there is variation in the ecological and physiological characteristics among the colour morphs of *H. axyridis*, offering particular morphs a greater fitness than others in specific habitats or at specific times (Osawa and Nishida 1992; Serpa et al. 2003; Wang et al. 2009; Berkvens et al. 2008a). Soares et al. (2001, 2005) and Berkvens et al. (2008b) found that red morphs are nutritionally more adaptive and that their greater nutritional plasticity may offer them a competitive advantage for the exploitation of food sources during establishment. Their greater nutritional plasticity combined with a greater dispersal potential may in part explain the predominance of non-melanic morphs in invaded areas (Koch 2003; Hantson 2004).
Arguably, flight mill experiments like those conducted in this study have their limitations. First, long-term laboratory rearing of natural enemies could induce selective adaptation on their flight propensity (Grenier and De Clercq 2003). Further, data obtained from flight mill experiments allow only for an estimation of flight capacity (Bruzzone et al. 2009). Because insects are forced to fly by lack of tarsal contact, flight mill experiments tend to overestimate their dispersal capacity compared with experiments carried out in flight chambers or mark-release-recapture experiments conducted at the field scale (Shirai and Kosugi 2000; Yamanaka et al. 2001; Blackmer et al. 2004; Botero-Garces and Isaacs 2004; Edwards 2006). Moreover, the handling of insects when attaching them to the flight mills can reduce or increase their propensity for flight (Cockbain 1961; Kennedy and Booth 1963). Besides, wind-assisted flight is obviously not measured with mills, which could lead to an underestimate of actual dispersal capacities. Nevertheless, recent comparative studies report consistent results between activity patterns measured in a flight mill and flight activity observed in the field (Amat et al. 2012). The use of computer-monitored flight mills has several advantages: flight mills are convenient and relatively inexpensive means to assess a species’ flight performance, the analysis of flight mill output data is simple and straightforward and flight mill experiments are less time consuming than traditional mark-release-recapture experiments which require repeated recaptures and releases over a period of time (Reynolds et al. 1997; Riley et al. 1997; Mills et al. 2006).

Although the calibration of flight mills to obtain absolute estimates of the dispersal capacity of an insect remains an important obstacle, flight mill experiments can yield significant information when used in a comparative approach. The present study indicated the strong flight capacity of the harlequin ladybird, *H. axyridis*, suggesting its role in the rapid invasion of Europe and other parts of the world. In the framework of an ERA procedure, candidate BCAs with such pronounced dispersal abilities would immediately be recognised in flight mill studies. Our experiments indicate that in view of standardizing such a risk assessment procedure, variability related to the mass rearing conditions of the studied BCA should not be ignored. We demonstrated that predatory ladybirds reared on *E.*
*kuehniella* eggs as factitious food outperformed ladybirds reared on natural prey. Likewise, we showed that the food source offered to *C. montrouzieri* influenced its supercooling capacity and might therefore affect its establishment potential (Chapter 5). These findings indicate that factors related to the rearing conditions of BCAs may complicate a risk assessment procedure and thus need to be taken into consideration.
Chapter 7

Laboratory prey range of *Cryptolaemus montrouzieri*

Redrafted after:

7.1 Introduction

The mealybug destroyer *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) is mainly released against mealybug pests, but it has also been reported to feed on a wide range of other hemipterans in the field, including aphids, scales and whiteflies (Malais and Ravensberg 2002; Ślipiński 2007; Finlay-Doney and Walter 2012b; Cock 2013). Its potential to develop and reproduce when offered these alternative prey, however, has hardly been investigated.

The study of a candidate BCA’s host range is a key element in the risk assessment process, because a lack of host specificity might lead to unacceptable risk if the agent establishes and disperses widely, whereas, a highly specific species is not expected to create serious risk even when it establishes (Loomans and van Lenteren 2005). In this chapter, the prey range of *C. montrouzieri* in the laboratory was investigated. We evaluated the development and reproduction of *C. montrouzieri* when offered eight candidate prey species in a no-choice design. In augmentative biological control, *C. montrouzieri* is mostly released as adults. Therefore, we also assessed the reproductive capacity of ladybirds that received one of the candidate prey during their adult life, but had been reared on a nutritionally suitable food source (eggs of the flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)) during their larval stages (Chapter 4).

7.2 Materials and Methods

7.2.1 Selection of candidate prey species

Following prey species were selected according to the criteria (a) phylogenetic relatedness with the target prey *P. citri*, (b) economic or ecological value and (c) availability for testing (i.e. their potential to be reared in the laboratory):

- tobacco aphid *Myzus persicae nicotianae* (Sulzer) (Hemiptera: Aphididae)
- pea aphid *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae)
- tobacco whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae)

- southern green stinkbug *Nezara viridula* (L.) (Hemiptera: Pentatomidae)

- western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae)

- two-spotted ladybird *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae)

- yellow mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)

- greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae)

As it is well documented that several members of the Pseudococcidae family are suitable prey for *C. montrouzieri* (Malais and Ravensberg 2002; Ślipiński 2007; Finlay-Doney and Walter 2012b; Kaur and Virk 2012), species from other families within the Hemiptera were considered for testing according to their descending degree of relatedness with Pseudococcidae (Bourgoin and Campbell 2002). The nearest families tested were the Aphididae and Aleyrodidae. *Myzus persicae*, *A. pisum* and *B. tabaci* were chosen as representatives for these families because they are known to cause economic crop damage. *Nezara viridula* was chosen as a representative for the Pentatomidae family, which is more distantly related to the Pseudococcidae but still belongs to the Hemiptera. Further, the western flower thrips *F. occidentalis* was selected as another economically important hemimetabolous pest insect. The ladybird *A. bipunctata*, native to the study area (Western Europe), was selected for its ecological value. *Tenebrio molitor* was chosen as a second representative for the order of the Coleoptera. Finally, the eggs of *G. mellonella* were tested as a potential food source for *C. montrouzieri* as it is closely related to *E. kuehniella*, the eggs of which constitute an adequate factitious food source for *C. montrouzieri* (Attia et al. 2011a; Chapter 4). An overview of the characteristics of the different candidate prey species is presented in Table 16.
Table 16: Overview of the characteristics (size, mobility, body texture and ecological function) of the candidate prey species tested for *C. montrouzieri*.

<table>
<thead>
<tr>
<th>Prey</th>
<th>Size (length in mm)</th>
<th>Mobility</th>
<th>Body texture</th>
<th>Ecological function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. persicae nicotianae</em> all stages</td>
<td>±1-2</td>
<td>Mobile</td>
<td>Soft</td>
<td>Herbivore</td>
</tr>
<tr>
<td><em>A. pisum</em> all stages</td>
<td>±2-4</td>
<td>Mobile</td>
<td>Soft</td>
<td>Herbivore</td>
</tr>
<tr>
<td><em>B. tabaci</em> nymphs</td>
<td>±0.4</td>
<td>Sedentary</td>
<td>Soft</td>
<td>Herbivore</td>
</tr>
<tr>
<td><em>N. viridula</em> eggs</td>
<td>±1.3</td>
<td>Immobile</td>
<td>Hard</td>
<td>Herbivore</td>
</tr>
<tr>
<td><em>F. occidentalis</em> nymphs</td>
<td>±0.5</td>
<td>Very mobile</td>
<td>Soft</td>
<td>Herbivore</td>
</tr>
<tr>
<td><em>A. bipunctata</em> eggs</td>
<td>±1.2</td>
<td>Immobile</td>
<td>Soft</td>
<td>Carnivore</td>
</tr>
<tr>
<td><em>T. molitor</em> eggs</td>
<td>±1.3</td>
<td>Immobile</td>
<td>Hard</td>
<td>Stored product pest</td>
</tr>
<tr>
<td><em>G. mellonella</em> eggs</td>
<td>±0.4</td>
<td>Immobile</td>
<td>Intermediate</td>
<td>Honeycomb feeder</td>
</tr>
</tbody>
</table>

### 7.2.2 Insect cultures

#### 7.2.2.1 Cryptolaemus montrouzieri

The laboratory colony of *C. montrouzieri* was reared on *E. kuehniella* eggs as described in section 5.2.1. Individuals of the 12th and 13th generations were used in the experiments.

#### 7.2.2.2 Unnatural prey species

A laboratory culture of the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) was initiated in 2010 with nymphs supplied by Koppert BV (Berkel en Rodenrijs, The Netherlands). The mealybugs were cultured on potato sprouts and kept in a dark room at ambient conditions. A mixture of all nymhal stages of *P. citri* was offered to *C. montrouzieri* in the feeding trials.

A colony of *M. persicae nicotianae* was started in 2012 with individuals provided by Koppert BV and maintained at ambient laboratory conditions on sharp pepper plants, *Capsicum annuum* L. Mixed stages of the peach aphid were offered to *C. montrouzieri* as a potential food source.

A laboratory population of *A. pisum* was established in 2013 using individuals obtained from Koppert BV. Pea aphids were fed on faba bean *Vicia faba* L. at ambient conditions. A mixture of all stages of *A. pisum* was used in the experiments.
Tobacco whiteflies were obtained from Koppert BV and reared on tobacco, *Nicotiana tabacum* L. Leaves containing second and third instar nymphs were offered to *C. montrouzieri*.

A laboratory colony of *N. viridula* was established in 1999 using insects originating from different field collections in France, Spain and Italy. The stinkbugs were fed on pods of green bean *Phaseolus vulgaris* L. and on seed kernels of sunflower *Helianthus annuus* L. and maintained in an incubator set at 23±1°C, 65±5% RH and a 16:8(L:D)h photoperiod. Only stinkbug eggs were offered as potential prey to *C. montrouzieri*.

A laboratory population of *F. occidentalis* was established in 2011 using insects collected on rose plants (*Rosa* spp.) in Belgian greenhouses. Thrips were cultured in plastic boxes containing vermiculite and green bean pods (*P. vulgaris*) and maintained in an incubator set at 23±1°C, 70±5% RH and a 16:8(L:D)h photoperiod. The second instar of the western flower thrips was tested as a prey for *C. montrouzieri*.

A population of *A. bipunctata* was initiated from specimens supplied by CRA-W (Gembloux, Belgium) in 2012 and kept in an incubator set at 23±1°C, 65±5% RH and a 16:8(L:D)h photoperiod. The ladybirds were fed a mixture of frozen *E. kuehniella* eggs and bee pollen, as described in section 7.2.1. The eggs of *A. bipunctata* were offered to *C. montrouzieri* as a food source.

A laboratory colony of *T. molitor* was initiated in 2013 with individuals obtained from Vivara (Vierlingsbeek, The Netherlands). The beetles were fed wheat flour and slices of apple. The eggs of the mealworm were tested as potential prey for *C. montrouzieri*.

A laboratory culture of *G. mellonella* was initiated in 1980 and reared at ambient conditions on an artificial diet described by Vanhaecke and Degheele (1980). Eggs were tested as prey for *C. montrouzieri*. 
7.2.3 Experimental set-up

In a first experiment, the potential of *C. montrouzieri* to develop and reproduce on the different test species was investigated by offering the ladybirds the same candidate prey during their larval and adult stages. Prey species that proved to be less suitable to support the development of *C. montrouzieri* were subjected to a second laboratory experiment, in which their potential to support the reproduction of *C. montrouzieri* was assessed. In the latter experiment, ladybird larvae were reared to adulthood on a diet of *E. kuehniella* eggs, which was found to be an adequate food source for *C. montrouzieri* larvae (Chapter 4), and switched to one of the candidate prey species once they reached the adult stage. This was done for all candidate prey species, except for the aphids *M. persicae* and *A. pisum*.

In both experiments, approximately 60 first instar *C. montrouzieri* larvae (<24h) per prey species (depending on availability of both ladybirds and prey) were taken out of the stock colony and placed individually in plastic Petri dishes (Ø: 9 cm, height: 2 cm). Water was provided by way of a moist wadding plug fitted into a 1.5 cm plastic dish. All foods were offered ad libitum and replenished every day, except for *E. kuehniella* eggs, which were replenished every two days. Survival and development of *C. montrouzieri* larvae was monitored daily. Newly emerged adults were sexed and weighed using a Sartorius Genius ME215P balance, after which they were randomly paired. The oviposition substrate (a piece of synthetic wadding (1x1cm)) was checked daily for eggs to determine the preoviposition period. Once the first egg was laid, substrates were replaced three times a week for a total period of 1 month. Oviposition rate and egg hatch were monitored during the first 30 days of egg laying. When offered *A. bipunctata* eggs during their larval development, only 2 females reached the adult stage. These were paired with newly emerged males from the stock colony in order to study their reproductive potential. In both series of experiments, a positive control treatment consisting of *P. citri* mealybugs and a negative control treatment consisting of water only were set up. All experiments were conducted in a climatic chamber set at 25±1°C, 75±5% RH, and a 16:8(L:D)h photoperiod.
7.2.4 Statistical analysis

All data were analyzed using SPSS 21.0 (SPSS Inc. 2009). Survival rates, egg hatch and sex ratio of the predators were considered as binary data, which enabled us to calculate standard errors on percentages. The means were compared by way of a logistic regression. This regression is a generalized linear model using a probit (log odds) link and a binomial error function (McCullagh and Nelder 1989). P-values below 0.05 were considered significant.

A Kolmogorov-Smirnov test indicated that male and female body weight of the predator were normally distributed and therefore analyzed using a one-way analysis of variance (ANOVA). The means were separated using Tukey tests, as a Levene test indicated homoscedasticity. Because male and female developmental times were not normally distributed (according to a Kolmogorov-Smirnov test), the non-parametric Kruskal-Wallis H test and Mann-Whitney U tests were used to evaluate differences in developmental time among treatments.

In all cases, total fecundity (i.e. the number of deposited eggs during a 30-day period) was found to be normally distributed (Kolmogorov-Smirnov test) and thus analyzed using ANOVA. While the ANOVA indicated no differences among treatments for experiment 1, the means in experiment 2 were separated using Tukey post-hoc tests, after the Levene test indicated homoscedasticity. The parameter preoviposition period was not normally distributed and thus analyzed using a non-parametric Kruskal-Wallis H test followed by Mann-Whitney U tests (SPSS Inc. 2009).

7.3 Results

Immature survival was significantly influenced by prey species ($\chi^2=147.25$, df=5, $P<0.001$)(Table 17).

Whereas survival was high when *C. montrouzieri* was fed on *P. citri* and *M. persicae* (93% and 81%, respectively), low on *A. pisum* and *B. tabaci* (both 20%) and very poor on *A. bipunctata* and *G. mellonella* eggs (3% and 2%, respectively), none of the larvae succeeded in reaching the adult stage
when offered *F. occidentalis* nymphs, *N. viridula* eggs or *T. molitor* eggs. For candidate prey species where *C. montrouzieri* survival was low or zero, mortality of the predator occurred during the first and second instars (Figure 22). Although total survival on *A. pism* and *B. tabaci* was similar, most larvae died early in their development (L1-L2) when offered *A. pism* but late in their development (L3-L4) when provided with *B. tabaci*. Only 2 individuals reached the pupal stage when offered eggs of *T. molitor*, another 2 individuals reached adulthood on *A. bipunctata* eggs and only 1 individual reached the adult stage on *G. mellonella* eggs.

**Table 17**: Development of *C. montrouzieri* fed different candidate prey species.

<table>
<thead>
<tr>
<th>Prey</th>
<th>N</th>
<th>Larval survival (%)</th>
<th>Developmental time (days)</th>
<th>Adult weight (mg)</th>
<th>Sex ratio (% females)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td><em>P. citri</em> nymphs</td>
<td>45</td>
<td>93.3 ± 0.04a</td>
<td>24.1 ± 0.2a</td>
<td>24.5 ± 0.3a</td>
<td>10.6 ± 0.2a</td>
</tr>
<tr>
<td><em>M. persicae</em> nymphs</td>
<td>59</td>
<td>81.4 ± 5.1a</td>
<td>26.3 ± 0.3b</td>
<td>27.1 ± 0.4b</td>
<td>7.7 ± 0.2b</td>
</tr>
<tr>
<td><em>A. pism</em> nymphs</td>
<td>59</td>
<td>20.0 ± 6.4b</td>
<td>31.0 ± 1.5c</td>
<td>30.2 ± 0.5c</td>
<td>7.4 ± 0.7b</td>
</tr>
<tr>
<td><em>B. tabaci</em> nymphs</td>
<td>58</td>
<td>19.9 ± 5.2b</td>
<td>40.0 ± 3.5c</td>
<td>39.4 ± 1.6d</td>
<td>4.6 ± 0.4c</td>
</tr>
<tr>
<td><em>A. bipunctata</em> eggs**</td>
<td>64</td>
<td>3.1 ± 2.2c</td>
<td>31.0 ± 1.0c</td>
<td>-</td>
<td>6.2 ± 0.9bc</td>
</tr>
<tr>
<td><em>G. mellonella</em> eggs***</td>
<td>60</td>
<td>1.7 ± 1.7c</td>
<td>-</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td><em>F. occidentalis</em> nymphs</td>
<td>60</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>N. viridula</em> eggs</td>
<td>60</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>T. molitor</em> eggs</td>
<td>51</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>water only</td>
<td>54</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Means ± SE within a column followed by the same letter are not significantly different (P>0.05; Probit analysis (Wald-Chi²) (survival, sex ratio), Mann Whitney U test (developmental time) or Tukey test (adult weight))

**only 2 females reached the adult stage

***only 1 male reached the adult stage

Prey had a significant effect on developmental time of both males (χ²=42.35, df=3, P<0.001) and females (χ²=39.48, df=4, P<0.001)(Table 17). When *C. montrouzieri* larvae were reared on *P. citri*, development was approximately 2 days faster than on *M. persicae*, 6 days faster than on *A. pism* and *A. bipunctata*, and 16 days faster than on *B. tabaci* (all P<0.001). Also adult body weight was influenced by prey (F=62.15, df=3, 60, P<0.001 for males; F=65.78, df=4, 51, P<0.001 for females). Predators provided with *B. tabaci* weighed approximately 40% less than those provided with *M. persicae* or *A. pism* and 60% less than those provided with *P. citri* (all P<0.001).
Preoviposition period was affected by prey species ($\chi^2=28.29$, df=2, $P<0.001$) (Table 18). Egg laying was postponed by 6 and 9 days when the ladybirds were fed with *M. persicae* and *A. pisum*, respectively, instead of *P. citri* (both $P<0.001$). No significant differences in total numbers of deposited eggs were found between ladybirds fed mealybugs or aphids ($F=1.65$, df=2, 46, $P=0.20$). Prey species also influenced the hatching rate of the eggs ($\chi^2=111.90$, df=4, $P<0.001$). A single female succeeded in producing viable eggs on *B. tabaci* and on *A. bipunctata*. The egg hatch rate observed on *B. tabaci* was, however, substantially higher than on *A. bipunctata* ($P<0.001$) and equalled that of females fed *P. citri* ($P=0.44$) or *M. persicae* ($P=0.15$).

**Figure 22**: Mortality in consecutive developmental stages (L1-Pupa) of *C. montrouzieri* fed different prey species.

**Figure 22**

<table>
<thead>
<tr>
<th>Prey</th>
<th>N</th>
<th>Preoviposition period (days)</th>
<th>No. of eggs laid per female in 30 days</th>
<th>Egg hatch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. citri</em> nymphs</td>
<td>21</td>
<td>4.1 ± 0.2a</td>
<td>154.3 ± 17.5a</td>
<td>67.5 ± 0.8a</td>
</tr>
<tr>
<td><em>M. persicae</em> nymphs</td>
<td>20</td>
<td>10.4 ± 0.9b</td>
<td>200.2 ± 19.3a</td>
<td>62.9 ± 0.8b</td>
</tr>
<tr>
<td><em>A. pisum</em> nymphs</td>
<td>4</td>
<td>13.5 ± 1.7b</td>
<td>161.5 ± 29.0a</td>
<td>54.6 ± 2.0c</td>
</tr>
<tr>
<td><em>B. tabaci</em> nymphs</td>
<td>3</td>
<td>24**</td>
<td>51</td>
<td>72.6 ± 6.3ab</td>
</tr>
<tr>
<td><em>A. bipunctata</em> eggs</td>
<td>2</td>
<td>8**</td>
<td>69</td>
<td>8.7 ± 3.4d</td>
</tr>
</tbody>
</table>

*Means ± SE within a column followed by the same letter are not significantly different ($P>0.05$; Mann-Whitney U test (preoviposition period), Tukey test (No. of oviposited eggs) or Probit analysis (Wald-$\chi^2$)(egg hatch))

**Table 18**: Reproduction of *C. montrouzieri* fed different candidate prey species. All predators were offered the same prey during their larval and adult stages (experiment 1).
Table 19 shows the reproduction parameters of *C. montrouzieri* ladybirds fed *E. kuehniella* eggs as larvae and different prey species as adults. No egg laying was observed for adults offered *G. mellonella, F. occidentalis, N. viridula, T. molitor* or water alone; in these treatments all females died within 15, 9, 11, 41 and 10 days, respectively. Preoviposition period on the remaining diets was affected by prey ($\chi^2=14.03$, df=2, $P=0.001$) and was nearly 5 days longer on *B. tabaci* ($P=0.02$) and *A. bipunctata* ($P<0.001$) than on *P. citri*. Also total fecundity ($F=9.87$, df=2, 52, $P<0.001$) and egg hatch ($\chi^2=816.44$, df=2, $P<0.001$) were influenced by diet. Females fed *A. bipunctata* laid significantly more eggs than those fed *B. tabaci* ($P=0.013$), but were less fecund than those reared on *P. citri* ($P<0.001$). Egg hatch, on the other hand, was higher for females fed *B. tabaci* nymphs than for those given *A. bipunctata* eggs ($P<0.001$), but was still lower than for females fed *P. citri* nymphs ($P<0.001$).

**Table 19:** Reproduction of *C. montrouzieri* fed *E. kuehniella* eggs as larvae and different candidate prey species as adults (experiment 2).

<table>
<thead>
<tr>
<th>Prey</th>
<th>N</th>
<th>Preoviposition period (days)</th>
<th>No. of eggs laid per female in 30 days</th>
<th>Egg hatch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. citri</em> nymphs</td>
<td>25</td>
<td>10.4 ± 0.9a</td>
<td>179.4 ± 17.0a</td>
<td>69.3 ± 0.7a</td>
</tr>
<tr>
<td><em>B. tabaci</em> nymphs</td>
<td>25</td>
<td>15.4 ± 0.6b</td>
<td>40.6 ± 16.5c</td>
<td>46.8 ± 2.9b</td>
</tr>
<tr>
<td><em>A. bipunctata</em> eggs</td>
<td>21</td>
<td>15.8 ± 0.9b</td>
<td>116.4 ± 16.7b</td>
<td>33.6 ± 1.0c</td>
</tr>
<tr>
<td><em>G. mellonella</em> eggs</td>
<td>25</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td><em>F. occidentalis</em> nymphs</td>
<td>25</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td><em>N. viridula</em> eggs</td>
<td>25</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td><em>T. molitor</em> eggs</td>
<td>25</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>water</td>
<td>25</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
</tbody>
</table>

*Means ± SE within a column followed by the same letter are not significantly different ($P>0.05$; Mann-Whitney U test (preoviposition period), Tukey test (No. of oviposited eggs) or Probit analysis (Wald-Chi²)(egg hatch))

### 7.4 Discussion

Host specificity is a key element in the risk assessment of a candidate BCA. A critical step in determining the host range of a natural enemy in the laboratory is the selection of the non-target species to be tested. van Lenteren et al. (2003) proposed a selection procedure for non-target
species based on the phylogenetic centrifugal method used for the evaluation of weed biocontrol agents. This procedure starts with testing non-target species that are closely related to the target and then progresses to species that are more distantly related to the target organism. If none of the non-target species is attacked, one can stop testing (Wapshere 1974; Lonsdale et al. 2001; van Lenteren et al. 2006b). In the present study, survival was high to moderate when *C. montrouzieri* was provided with prey species that are closely related to the mealybug target prey (*M. persicae, A. pisum, B. tabaci*) and overall poor to zero when the ladybird was provided with prey species that belong to a different insect order than the Hemiptera (*F. occidentalis, A. bipunctata, T. molitor, G. mellonella*) or even hemipteran prey from a different suborder (*N. viridula*). Also the reproductive capacity of *C. montrouzieri* ladybirds decreased when they were provided with more distantly related prey species. While ladybirds reared on aphids during their development and adult life deposited similar numbers of eggs as their counterparts fed on *P. citri*, fecundity was markedly lower when the ladybirds were presented with whitefly and ladybird prey, and no eggs were laid in the presence of the other prey species.

However, predicting a predator’s prey range solely based on phylogenetic relatedness to the target prey may not be straightforward and the outcome may depend on the species selected for testing. Eggs of *G. mellonella* were not a suitable food source for *C. montrouzieri*: only 2% of the larvae reached the adult stage when fed *G. mellonella* eggs and not a single female produced eggs when provided with this food source. In contrast, the eggs of another member of the Pyralidae family (*E. kuehniella*) were found to be a suitable factitious food source for both development and reproduction of this ladybird (Attia et al. 2011; Chapter 4). This might be related to the nutritional composition (amino acids, fatty acids) of *E. kuehniella* eggs (Specty et al. 2003). Although *M. persicae* and *A. pisum* belong to the same family of Aphididae, *C. montrouzieri* performed differently on these aphid species. Whereas reproductive capacity and adult body weight were not affected by the aphid prey species, development was 3 days shorter and survival was 4 times higher when the predator was provided with *M. persicae* instead of *A. pisum*. Hence, both aphid species can be defined as
‘essential’ prey for *C. montrouzieri* as they both support full development and reproduction (Hodek and Honěk 1996), but *M. persicae* proved to be a more adequate prey for the ladybird than *A. pisum*. These findings indicate that taxonomic relatedness *in se* may not necessarily be a sufficiently reliable criterion for determining prey ranges and even closely related prey may substantially differ in their suitability to support immature development and/or reproduction of a natural enemy. Furthermore, our study provides support for the hypothesis that in addition to non-target species that can easily be tested in a laboratory setting, prey range testing should give additional attention to economically important species, threatened or valued species and native natural enemies (Sands and Van Driesche 2000; Babendreier et al. 2005; van Lenteren et al. 2006b).

A major practical concern in the evaluation of the host range of a candidate BCA is the number of non-target species that needs to be tested. Eventually, this will determine the practical feasibility of the proposed risk assessment procedure to be followed by commercial biocontrol producers who wish to place a new species on the market. Kuhlmann et al. (2006) suggested to design an initial test list with 50 non-target species and reduce this list to 10-20 species by the application of criteria filters such as ecological similarity and phenological overlap with the target prey. Based on the findings of the present study, it should be possible to perform a quick scan with a limited number of non-target prey to highlight those species that are potentially at risk and deserve the focus of the prey range testing. Our results indicate that the focus of prey range tests for *C. montrouzieri* should be on small, less mobile and soft-bodied prey species. Despite several feeding attempts on eggs of the stinkbug *N. viridula*, the large majority of eggs was not consumed, which might indicate that *C. montrouzieri* has difficulty handling prey materials characterized with a rigid texture and prefers soft-bodied organisms. The mobility of thrips larvae was deemed responsible for the low predation rate of *C. montrouzieri* on *F. occidentalis*, suggesting that the predator is adapted to less mobile prey like mealybugs. Also the body size of a test species might determine its suitability as prey for a predator. Over 80% of the *C. montrouzieri* larvae reared on *M. persicae* aphids successfully completed development, whereas only 20% of the larvae reared on *A. pisum* aphids reached the adult stage,
with the highest mortality being observed during the first and second instar. Similar survival rates during the third and fourth instar and similar reproductive capacity might indicate that not the biochemical properties of *M. persicae* but rather its smaller size are responsible for the better performance of *C. montrouzieri* on *M. persicae* than on *A. pisum*. However, it cannot be excluded that tritrophic effects caused by the different host plant-prey associations and experimental conditions may have affected the outcome of the experiments. For instance, whereas some prey were offered on paper or without a substrate, others were presented on plant materials. Besides the indirect effects of the host plant on the predator through prey quality, predation capacity and fitness of the predator may also have been directly affected by the presence or absence of plant materials in the test arenas. In addition, it is worth noting that small scale laboratory experiments do not take into account prey location cues used by the predator in the field (Finlay-Doney and Walter 2012a).

The selection of life history parameters to quantify the suitability of a non-target prey is another important aspect of a host range testing procedure for candidate BCAs (van Lenteren et al. 2003). In the present study, the parameters proposed by van Driesche and Murray (2004a) were monitored: feeding activity of larvae and adults, larval development, adult survival and oviposition. The investigation of multiple parameters in our experiments was critical as each parameter revealed additional information. For instance, prey species that were found to be less suitable for development and reproduction of *C. montrouzieri* could still be an adequate food source to sustain adult survival. This is probably due to the predator’s different nutritional requirements during its larval stages and adult life (Michaud 2005). Adult ladybirds survived for 41 days when fed *T. molitor* eggs and 85% of the adults provided with *A. bipunctata* eggs was still alive after 65 days. Eggs of *T. molitor* can therefore be defined as an ‘alternative’ food for *C. montrouzieri* as they can serve as a source of energy for the predator and thus increase survival when essential foods are not available (Hodek and Honék 1996). Besides, the relationship between development and survival on the one hand and reproduction on the other was not always straightforward. Whereas survival rates of *C. montrouzieri* on *A. pisum* and *B. tabaci* were similar, adult females laid 70% more eggs on *A. pisum*
than on *B. tabaci*. Finally, our experiments indicate that it is worth investigating a predator’s reproductive capacity on a certain candidate prey even when the larvae had difficulty to complete their development on this prey. Despite the fact that only 8% of the *C. montrouzieri* larvae reached the adult stage when provided with *A. bipunctata* eggs, females that had developed on *E. kuehniella* eggs and were supplied with *A. bipunctata* eggs from the adult stage on, were able to produce an average of 116 eggs in 30 days. This is 35% less than females provided with *P. citri* mealybugs, but is 35% more than females supplied with *B. tabaci* larvae, which proved to be a more suitable prey for larval development of *C. montrouzieri*. The suitability of *A. bipunctata* eggs for the reproduction and survival of adult ladybirds shows that *C. montrouzieri* might act as an intraguild predator. Previous studies pointed out that the mealybug destroyer may dominate the coccidophagous guild. Chong and Oetting (2007) found that both ladybird larvae and adults display a similar preference for healthy mealybugs and mealybugs parasitized by the *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae). Also Muştu et al. (2008) reported that *C. montrouzieri* does not discriminate between mealybugs parasitized or not by *Anagyrus pseudococci* (Girault)(Hymenoptera: Encyrtidae).

Arguably, laboratory experiments exploring the prey range of a predator like those conducted in the present study have their limitations. First, long-term rearing of natural enemies could induce selective adaptation to the food source offered in the laboratory and could result in natural enemies that have lost their ability to feed on some of their natural prey (Grenier and De Clercq 2003), which might lead to an underestimation of the prey range. Also, the potential of *C. montrouzieri* to reproduce in the absence of mealybugs might have been overestimated due to the experimental methods used. Adult ladybirds were always provided with a polyester wadding as an (artificial) oviposition substrate, which mimics the physical properties of mealybug egg masses and fulfils the requirements to trigger egg laying in *C. montrouzieri* (Chapter 4). Furthermore, no-choice experiments present a worst-case scenario as a positive response to a non-target prey can be artificially induced by confinement and lack of choice (Van Driesche and Murray 2004b). Conducting more realistic experiments, in which two or more prey species are presented to the predator (choice
test) or host plants are included in the experimental set-up (semi-field test), might yield a more reliable estimation of a predator’s prey range (van Lenteren et al. 2003; van Driesche and Murray 2004b; Babendreier et al. 2005). Thus, test species that showed to be suitable prey for *C. montrouzieri* in the present no-choice Petri dish experiments do not necessarily have to be at risk in a natural situation. On the other hand, negative results observed in the current study indicate that *C. montrouzieri* is not likely to use these species as a field prey. However, it cannot be excluded that the predator may be able to use this prey as part of a mixed diet, as many predators appear to benefit from mixed diets as compared to certain single-species diets (Lefcheck et al. 2013). Van Driesche and Murray (2004b) noted that a predator may in fact not have a choice of prey species if it expands geographically beyond the range of its target pest, if it invades habitats not occupied by the target pest, if the predator is partially out of synchrony with its target pest, or if the target pest is absent for any other reason (including biological control itself and chemical control). In our no-choice reproduction experiments, a single female each time was able to produce viable eggs on *B. tabaci* nymphs or *A. bipunctata* eggs. It should be noted that when confronted with a lack of choice, a strong selection in favour of the few females able to reproduce on alternative prey could occur. Conducting multiple generation experiments on candidate prey can help to understand this mechanism.

In conclusion, our laboratory study indicates that the prey range of *C. montrouzieri* is not limited to the Pseudococcidae, but includes other small, soft-bodied and sedentary hemipterans. To a lesser extent, also eggs of coleopterans and lepidopterans supported survival, larval development and/or reproduction of the ladybird. Whereas there were only scattered reports of the feeding on non-mealybug prey by *C. montrouzieri* in the literature (Kairo et al. 2012; Finlay-Doney and Walter 2012b), the present study compared the effects of non-mealybug prey from different insect orders on the developmental and reproductive performance of the predator. Although we observed a reduced fitness of the predator when offered non-mealybug prey species, our data indicate that it may be able to sustain itself in a crop on alternative prey when mealybugs are absent or mealybug...
populations are low. Considering the ladybird’s potential to develop and reproduce on *M. persicae*, it may to some extent contribute to the suppression of this aphid pest, but this needs to be confirmed in the field. On the negative side, the non-specific feeding habit of *C. montrouzieri* increases the risk that the predator will attack non-target prey. In areas where the predator cannot establish because of its limited cold tolerance, the effect of its oligophagous feeding behaviour on populations of non-target organisms is expected to be transient. However, in warmer climates its non-specific feeding behaviour may affect the local distribution of non-target prey in both agricultural and natural ecosystems.
The influence of acclimation, endosymbionts and diet on the supercooling capacity of *Macrolophus pygmaeus*

Redrafted after:

8.1 Introduction

*Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) is widely distributed in Europe and has been reported from the Mediterranean Region up to Finland (Kerzhner and Josifov 1999; Fauna Europaea 2011). However, the population that became widely commercially available for biological control in protected cultivation in northwestern Europe originates from Mediterranean Europe (J. Klapwijk, Koppert BV, pers. comm.).

The establishment potential of the biocontrol population of *M. pygmaeus* was assessed by determining the supercooling point (SCP), i.e. the temperature at which the insect’s body fluids freeze (Lee 1989). This parameter provides a first indication of an insect’s establishment potential in a new region and of its possible geographical range. The influence of acclimation, infection with endosymbiotic bacteria and diet on the SCP of the commercially available population of *M. pygmaeus* was assessed. In the laboratory *M. pygmaeus* is usually cultured under continuous summer conditions, whereas insects in the field are confronted with temperatures that vary with the season (Block 1990; Danks 2005). Because field insects may become acclimatized to colder conditions as temperatures gradually drop in autumn, exposing laboratory cultured *M. pygmaeus* directly to low temperatures may lead to inaccurate predictions of their cold tolerance. Finally, diets used in commercial insectaries may have a strong impact on the physiological responses of the natural enemies produced (Grenier and De Clercq 2003) and may thus also influence their responses to climatic conditions. Therefore, the freezing point of *M. pygmaeus* adults offered an artificial diet based on egg yolk was compared to those reared on a factitious food source, eggs of the flour moth *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae).
Supercooling point of *M. pygmaeus*

8.2 Materials and Methods

8.2.1 Treatment groups

A stock colony of *M. pygmaeus* was established in 2006 with eggs acquired from Koppert BV (Berkelen Rodenrijs, The Netherlands). The insects were reared in Plexiglas cylinders (Ø: 9 cm, height: 4 cm) and kept at 23°C, 65 ± 5% relative humidity and a photoperiod of 16:8 (L:D)h. Each cylinder contained a small bell pepper plant (*Capsicum annuum* L. Cv. California Wonder) as an oviposition substrate and a source of moisture. Frozen *E. kuehniella* eggs were supplied three times a week as a food source.

Four different treatment groups of *M. pygmaeus* were created to test whether their infection status on the one hand or diet on the other hand affected their supercooling abilities. Table 20 summarizes the properties of these treatment groups.

**Table 20**: Characteristics of the different *M. pygmaeus* treatment groups tested: infection status (infected with or cured of endosymbionts) and diet offered (*E. kuehniella* eggs or artificial egg yolk diet).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Infection status</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Cured</td>
</tr>
<tr>
<td>IE</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>CY</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>CE</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

A first treatment group, IE, was naturally infected with the endosymbiotic bacteria *Wolbachia pipiensis*, *Rickettsia limoniae* and *R. bellii* (Machtelinckx et al. 2012) and was fed with *E. kuehniella* eggs. Treatment group IV was established by offering insects taken from the stock colony an artificial diet based on egg yolk (Vandekerkhove et al. 2006)(Figure 23). Treatment group CY was created by curing insects taken from the stock colony of their endosymbiotic bacteria by feeding them the same egg yolk based artificial diet as population IV, but now supplemented with 0.1% tetracycline (Vandekerkhove et al. 2006; Machtelinckx et al. 2009). A fourth treatment group, CE, was also cured of endosymbionts and reared as described for treatment group CY. From generation 13 on, however, the insects were fed with frozen *E. kuehniella* eggs and no longer received the tetracycline treated...
diet. Taxonomic identity and infection status of the different treatment groups was confirmed with PCR diagnostics prior to testing (Machtelinckx et al. 2009; Machtelinckx et al. 2012). Forty acclimated and non-acclimated adult *M. pygmaeus* (both males and females in a 1:1 proportion) were tested to determine the supercooling points of each treatment group.

![Diagram](image)

**Figure 23:** Different treatment groups of *M. pygmaeus* created with insects springing from the same stock colony (naturally infected with endosymbionts and fed *E. kuehniella* eggs).

### 8.2.2 Acclimation

The supercooling capacity was tested for non-acclimated individuals and for individuals that were acclimated to lower temperatures. Non-acclimated insects were maintained at 23°C, 65 ± 5% relative humidity and a photoperiod of 16:8 (L:D)h. Newly emerged adults of the different populations were allowed to feed for 24h on their respective diets before testing. To acclimate insects to lower temperatures, newly moulted adults were placed in Plexiglas cylinders containing a bell pepper plant. After allowing the adults to feed for 24h, the cylinders were transferred to incubators set at 10°C and a photoperiod of 16:8(L:D)h for 7 days. *Ephestia kuehniella* eggs or domes containing egg yolk diet were available throughout the acclimation period.

### 8.2.3 Measurement of supercooling point

The freezing point was measured using a Picotech TC-08 thermocouple datalogger and a low temperature programmable Haake Phoenix II CP30 alcohol bath (see section 5.2.3). Each
Supercooling point of *M. pygmaeus*

A thermocouple was led individually through the lid of a 1.5 ml Eppendorf tube. The insects were attached to the thermocouples with petroleum jelly and the Eppendorf tubes were sealed with Pritt Poster Buddy. The Eppendorf tubes were placed individually in glass tubes which were immersed in the alcohol bath (Berkvens et al. 2010). The starting temperature was set at 23°C (rearing temperature) or 10°C (acclimation temperature). The insects were cooled at 0.5°C/min until the thermocouple registered the release of exothermic heat, at which point the supercooling temperature is reached. Before the insects were subjected to the experiments, they were weighed using a semi-microbalance Sartorius Genius ME215P (Sartorius AG, Goettingen, Germany) (±0.01mg) in order to check whether supercooling ability was correlated to body weight.

### 8.2.4 Statistical analysis

A Kolmogorov-Smirnov test indicated that the SCP values were normally distributed. SCP values were analyzed using a four-way analysis of variance (ANOVA) with following factors: acclimation, endosymbionts, diet and gender. The means were separated using Tukey or Tamhane tests, when a Levene test indicated homoscedasticity or heteroscedasticity, respectively. P-values below 0.05 were considered significant. The relationship between body weight and SCP was assessed with a Pearson’s correlation test. All data were analyzed using SPSS 16.0 (SPSS Inc. 2009).

### 8.3 Results

A four-way analysis of variance (ANOVA) showed no four- or three-factorial interactions between the factors acclimation, endosymbionts, diet and gender for the SCP (Table 21). However, the interaction endosymbionts x gender was significant (P=0.012), indicating that the gender of *M. pygmaeus* influenced the effect of endosymbionts on the SCP. No other two-way interactions were detected. The main effects acclimation (P<0.001), endosymbionts (P<0.001), diet (P<0.001) and gender
(P=0.029) all had a significant impact on SCP. Overall, acclimation led to an increase in supercooling capacity of *M. pygmaeus*. On the other hand, offering the bugs an artificial diet instead of *E. kuehniella* eggs led to a decrease in cold tolerance.

**Table 21**: Four-way ANOVA results indicating the effect of acclimation, diet, endosymbionts and gender on SCP of *M. pygmaeus*.

<table>
<thead>
<tr>
<th>Factor</th>
<th>F</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acclimation</td>
<td>18.187</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diet</td>
<td>17.342</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endosymbionts</td>
<td>66.191</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender</td>
<td>4.813</td>
<td>1</td>
<td>0.029</td>
</tr>
<tr>
<td>Acclimation x diet</td>
<td>0.060</td>
<td>1</td>
<td>0.807</td>
</tr>
<tr>
<td>Acclimation x endosymbionts</td>
<td>1.250</td>
<td>1</td>
<td>0.264</td>
</tr>
<tr>
<td>Acclimation x gender</td>
<td>1.172</td>
<td>1</td>
<td>0.280</td>
</tr>
<tr>
<td>Diet x endosymbionts</td>
<td>3.844</td>
<td>1</td>
<td>0.051</td>
</tr>
<tr>
<td>Diet x gender</td>
<td>1.099</td>
<td>1</td>
<td>0.295</td>
</tr>
<tr>
<td>Endosymbionts x gender</td>
<td>6.392</td>
<td>1</td>
<td>0.012</td>
</tr>
<tr>
<td>Acclimation x diet x endosymbionts</td>
<td>1.081</td>
<td>1</td>
<td>0.299</td>
</tr>
<tr>
<td>Acclimation x diet x gender</td>
<td>0.009</td>
<td>1</td>
<td>0.924</td>
</tr>
<tr>
<td>Acclimation x endosymbionts x gender</td>
<td>2.319</td>
<td>1</td>
<td>0.129</td>
</tr>
<tr>
<td>Diet x endosymbionts x gender</td>
<td>0.141</td>
<td>1</td>
<td>0.707</td>
</tr>
<tr>
<td>Acclimation x diet x endosymbionts x gender</td>
<td>1.146</td>
<td>1</td>
<td>0.702</td>
</tr>
<tr>
<td>Error</td>
<td>-</td>
<td>302</td>
<td>-</td>
</tr>
</tbody>
</table>

Because of the two-way interaction between endosymbionts and gender, data were split and analyzed for males and females separately (Figure 24). Although a trend was visible that acclimated bugs were more cold hardy than non-acclimated ones, this difference was not significant for females (F=1.904; df=1, 257; P=0.170) or males (F=1.879; df=1, 157; P=0.173). Both males and females cured of their endosymbionts had a significantly lower SCP than infected males (F=11.893; df=1, 157; P=0.001) and females (F=59.494; df=1, 157; P<0.001). The effect of curing was twice as strong for females than for males, with a drop in crystallization temperature of 2.4°C and 1.2°C, respectively. Offering the insects an artificial diet based on egg yolk rather than *E. kuehniella* eggs led to a raise in
Supercooling point of *M. pygmaeus* SCP, a difference that was marginally significant for males (F=3.493; df=1, 157; P=0.063), but significant for females (F=13.186; df=1, 157; P<0.001).

**Figure 24**: Influence of acclimation (white = non-acclimated, grey = acclimated), endosymbionts (white = infected, grey = cured) and diet (white = *E. kuehniella* eggs, grey = egg yolk diet) on the SCP of *M. pygmaeus* males (M) and females (F). Within each factor, graph bars (means ± SE) with the same letter are not significantly different (P > 0.05; Tukey test).

Acclimated insects of treatment group CE were found to be most cold tolerant with SCP values of -19.5 ± 0.3°C and -19.1 ± 0.2°C for males and females, respectively (Table 22). At the other side of the
spectrum, non-acclimated bugs of treatment group IY were observed to freeze already at -16.5 ± 0.5°C (males) and -15.1 ± 0.5°C (females). Insects of the other treatment groups had intermediate SCP values.

### Table 22: Mean SCP of (non)-acclimated *M. pygmaeus* males and females, cured from or infected with endosymbionts and fed with *E. kuehniella* eggs or an artificial egg yolk diet.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>N</th>
<th>SCP (°C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>CE, acclimated</td>
<td>40</td>
<td>-19.1 ± 0.2Aa</td>
</tr>
<tr>
<td>CE, non-acclimated</td>
<td>40</td>
<td>-18.8 ± 0.4Aa</td>
</tr>
<tr>
<td>CY, acclimated</td>
<td>38</td>
<td>-18.8 ± 0.3Aa</td>
</tr>
<tr>
<td>CY, non-acclimated</td>
<td>38</td>
<td>-17.8 ± 0.4Aab</td>
</tr>
<tr>
<td>IE, acclimated</td>
<td>41</td>
<td>-17.6 ± 0.5Aab</td>
</tr>
<tr>
<td>IE, non-acclimated</td>
<td>41</td>
<td>-16.5 ± 0.4Abc</td>
</tr>
<tr>
<td>IY, acclimated</td>
<td>39</td>
<td>-15.6 ± 0.4Ac</td>
</tr>
<tr>
<td>IY, non-acclimated</td>
<td>41</td>
<td>-15.1 ± 0.5Ac</td>
</tr>
</tbody>
</table>

*Means ± SE; means within the same column or row followed by the same lowercase or capital letter, respectively, are not significantly different (P>0.05; Tamhane test)

Adult body weights ranged from 0.62 ± 0.03 (treatment group IY, non-acclimated) to 0.76 ± 0.07mg (treatment group CY, acclimated) for males and from 1.13 ± 0.05 (treatment group CY, acclimated) to 1.31 ± 0.07mg (treatment group CY, acclimated) for females. Weights did not differ significantly among treatment groups for males (F=1.367; df=7, 151; P=0.223) but were significantly affected by acclimation in the females (F=8.585; df=1, 3; P=0.004). Females acclimated for 7 days to lower temperatures were heavier than 1-day old non-acclimated females coming directly out of the stock culture. There was no significant correlation between body weight and SCP for males (n=159, r=-0.117, P=0.117) or females (n=159, r=-0.135, P=0.091).

### 8.4 Discussion

Formerly, the SCP was generally considered to be the lowest lethal temperature of freeze-intolerant insects and it was expected that exposure to temperatures above the SCP would be survived (Salt
Supercooling point of *M. pygmaeus*

1966). This theory was refined by Bale (1993, 1996) who pointed out that the majority of freeze-avoiding species may die before the SCP is reached as a result of cumulative chilling injury. Although the SCP should not be considered as the only measure to determine the cold tolerance of insects, species inhabiting temperate and polar climates depend mostly on their supercooling ability to overwinter (Sømme 1989; Duman et al. 1991; Bale 2002). Therefore the SCP was used in this study as a first index of cold hardiness.

Factor-analysis showed that acclimation had a significant impact on the SCP (Table 21). Despite a trend towards lower SCP values for acclimated individuals in each *M. pygmaeus* treatment group tested, cold acclimation only significantly affected the SCP of cured males fed *E. kuehniella* eggs (Table 22). Several studies have demonstrated that enzyme activity, the production of antifreeze and thermal hysteresis proteins as well as alterations in the membrane lipid composition are triggered by low temperatures, even after a short cold exposure (Baust and Lee 1981; Storey and Storey 1988; Block 1990; Overgaard et al. 2005). However, Hart et al. (2002a) found no acclimation response for the predatory bug *Macrolophus caliginosus* Wagner (Hemiptera: Miridae) when measuring the SCP, but reported a trend in which acclimated adults were more cold hardy than non-acclimated adults based on the lower lethal temperature (i.e., the temperature at which 50% of the test individuals die) and lethal time (i.e., the time required to kill 50% of the population at a certain temperature) in exposures at 0°C and -5°C. For the parasitoid *Eretmocerus eremicus* Rose and Zolnerowich (Hymenoptera: Aphelinidae), the predatory bug *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) and the predatory mites *Typhlodromips montdorensis* (Schicha) and *Neoseiulus californicus* (McGregor) (Acari: Phytoseiidae) a significant decrease in SCP was not detected either after acclimation (Hart et al. 2002b; Tullett et al. 2004; Hatherly et al. 2004; Hughes et al. 2009). The present study used a similar experimental approach as abovementioned studies, in which the SCP of 1-day-old non-acclimated adults was compared with that of adults acclimated for 3 days (*E. eremicus*) or 7 days (*T. montdorensis, N. californicus, N. tenuis*). Hence, acclimated individuals were older than non-acclimated ones when the SCP was determined. Bowler and Terblanche (2008) noted
that low temperature tolerance may vary within a life stage. For example, for the fruit flies *Dacus tryoni* (Froggatt)(Diptera: Tephritidae) and *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) a decline in cold tolerance has been observed over their adult life (Meats 1973; David et al. 1998; Jensen et al. 2007). Likewise, the cold tolerance of *M. pygmaeus* could be affected by physiological changes induced by reproductive maturation or ageing. The divergence in age between acclimated and non-acclimated individuals could also have consequences for the bacterial load of both the digestive and reproductive system of *M. pygmaeus* and consequently for the total concentration of ice nucleating agents in the insect’s body.

When *M. pygmaeus* was cured from its infection with the endosymbiotic bacteria *W. pipientis*, *R. limoniae* and *R. bellii* the SCP decreased (Figure 24). This was most apparent for predators fed the artificial diet, where acclimated infected females had an SCP of -15.6°C, versus -18.8°C for uninfected females (Table 22). This increase in supercooling ability after elimination of bacterial symbionts could be due to the fact that these bacteria may act as heterogeneous ice nucleators inside the body of their insect host. Besides, feeding the predators a diet supplemented with antibiotics may have affected the bacterial load in the digestive system and may thus have further eliminated potential nucleating agents. The divergence in SCP found between the treatment group fed artificial diet and that fed the same artificial diet supplemented with antibiotics may thus does not only be attributed to the elimination of abovementioned endosymbionts by the antibiotics, but also to their effects on the gut bacteria. In earlier studies, ice nucleating active bacteria were isolated from the gut of the beetles *Hippodamia convergens* (Say)(Coleoptera: Coccinellidae) and *Cerotoma trifurcata* (Forster)(Coleoptera: Chrysomelidae). The bacteria were identified as *Enterobacter agglomerans* and *Enterobacter taylorae* and their activity was confirmed by feeding them to overwintering adults. Furthermore, a correlation was found between the concentration of ice nucleating active bacteria ingested and the degree in SCP elevation (Strong-Gunderson et al. 1990a,b; Lee et al. 1991). Worland and Block (1999) reported the occurrence of fluorescent *Pseudomonas* species in the gut of the beetles *Hydromedion sparsutum* (Müller)(Coleoptera: Perimylopidae) and *Perimylops antarcticus*
Supercooling point of *M. pygmaeus* (Müller)(Coleoptera: Perimylopidae). The authors assumed that nucleation of *Pseudomonas* at relatively high subzero temperatures in the gut was responsible for nucleation of the whole beetle. Tanaka (2001) noticed a dissimilarity in SCP of the house spider *Achaearanea tepidariorum* (Koch)(Araneae: Theridiidae) fed laboratory-reared prey and field-collected prey. Spiders offered field-collected prey had a higher, less negative SCP than those given laboratory-reared prey. It was suggested that field-collected prey contains efficient (external) ice nucleators, probably ice nucleating active bacteria, whereas laboratory animals may be less contaminated. The ice nucleating activity of the bacteria mentioned above is an extracellular process within the gut of the insect host. The endosymbiotic bacteria *Wolbachia* and *Rickettsia*, however, were observed to be intracellular in the reproductive tissues (*Wolbachia*) or in both the reproductive tissues and digestive system (*Rickettsia*) of *M. pygmaeus* (Machtelinckx et al. 2009; Machtelinckx et al. 2012). Testing the ice-nucleating activity of *Wolbachia* and *Rickettsia* by the droplet freezing assay of Vali (1971) is complicated by the obligate and intracellular nature of these endosymbionts which makes it impossible to culture them outside their insect host. Assuming that the bacteria can indeed act as heterogeneous ice nucleators, intracellular freezing will occur at higher temperatures when mirid bugs are infected with endosymbionts. This hypothesis is consistent with the decrease in supercooling ability detected in this study. The infection of *M. pygmaeus* with the endosymbiotic bacteria *Wolbachia* and *Rickettsia* may consequently limit the insect’s freezing tolerance. Several studies are dedicated to the capacity of these endosymbionts to alter the reproduction and fitness of their arthropod hosts (Werren 1997), but their potential to reduce the cold tolerance of their host and thus limit the host’s ecological barriers has not been reported before and needs further substantiation. However, there is increasing evidence that the infection status of a natural enemy with endosymbionts may affect the outcome of a biological control program (Zindel et al. 2011). It may thus be warranted to consider infection status of an invertebrate BCA in an ERA procedure.

Another factor affecting the supercooling ability of *M. pygmaeus* is the food offered to the bugs during their development. Overall, predators reared on *E. kuehniella* eggs had a lower SCP than those
fed the artificial diet based on egg yolk (Figure 24). Body weight plays an important role in insect freezing tolerance with smaller insects generally freezing at lower temperatures (Angell 1982; Sømme 1982; Johnston and Lee 1990). In this study, however, body weight was not influenced by diet. In contrast, Vandekerkhove et al. (2006) reported a significantly lower weight for *M. pygmaeus* bugs fed the same artificial diet than those fed *E. kuehniella* eggs. However, after rearing *M. pygmaeus* for 11 generations on the artificial diet adaptation to this diet could have occurred leading to higher body weights (Coudron et al. 2002). The differences in supercooling ability of *M. pygmaeus* fed different diets might be due to the different biochemical composition of these diets. Differences in nutritional composition of prey may be reflected in the biochemical composition of a predator’s body (Vandekerkhove et al. 2009). Biochemical analysis conducted on *E. kuehniella* eggs proved that the eggs are rich in fatty acids and amino acids (Specty et al. 2003). These nutrients may protect *M. pygmaeus* against extreme temperatures by delivering components that promote winter survival. For example, the production of antifreeze proteins may prevent freezing across the cuticle and promote supercooling by inhibiting ice crystal growth. Also the production of glycogen, triacylglycerol-1es and proline may protect insects against cold temperatures as these components may act as energy reserves and play a role in stabilizing cell membranes (Doucet et al. 2009).

The gender of *M. pygmaeus* was found to be correlated with the factor endosymbionts (Table 21). The increase in supercooling capacity as a result of curing the infection was shown to be twice as high for females than for males. Such differences may be related to a higher bacterial density in female ovaries than in male testes of *M. pygmaeus*, but this needs further confirmation by way of quantitative PCR analysis.

The results obtained in the present study may contribute to the development of an ERA scheme for exotic BCAs. Evaluating the establishment potential is considered to be one of the cornerstones of an ERA for a non-native arthropod BCA (van Lenteren et al. 2003; Bale 2011a). Hart et al. (2002a) identified three laboratory indices for winter survival: supercooling point, lethal temperature and
lethal time. A strong correlation was found between the lethal time at 5°C and the maximum field survival of different BCAs (Hatherly et al. 2005; Bale 2011a). Although the correlation between field survival and SCP was observed to be less strong (Hatherly et al. 2005), the latter parameter can be useful for a quick scan of an insect’s cold tolerance. Measuring the supercooling point takes no more than a few hours and requires only a limited number of test organisms. Our results indicate that acclimation period, infection status with endosymbionts and diet may influence the supercooling ability of a predatory insect, thus complicating the evaluation of its establishment potential in the framework of an ERA. We showed that insects that were allowed to acclimate to lower temperatures would be more likely to survive winter conditions than insects taken directly from the stock colony. On the other hand, exposing the predator to antibiotics and thus curing it from its infection with endosymbionts also increased cold tolerance and may eventually contribute to its establishment potential. In view of standardizing protocols for ERA testing, variability springing from different factors related to the rearing of the studied population need to be taken into consideration. Besides, exposing the predator to antibiotics and thus curing it from its infection with endosymbionts increased cold tolerance and may eventually contribute to its establishment potential. As natural populations may differ in their infection status with endosymbionts (Machtelinckx et al. 2012), our study provides support to conduct an ERA a population level rather than at a species level (Loomans and van Lenteren 2005).
Chapter 9

General discussion, conclusions and future perspectives
9.1 Implications for a regulation on exotic biological control agents

Although more than 2600 species of exotic natural enemies have been introduced to control plant or insect pests over the past 120 years, the majority of these releases did not result in unwanted side effects (Cock et al 2010). Nonetheless, some serious cases of non-target effects by exotic invertebrate BCAs against insects and weeds have been reported in recent years (Follett and Duan 2000; Wajnberg et al. 2000; van Lenteren 2012). An appropriate regulation concerning the import and use of natural enemies, based on a scientific ERA, is instrumental in preventing such non-target effects (van Lenteren et al. 2003; Bale 2011a; Ehlers 2011).

One of the main challenges in regulating the use of exotic BCAs is to install a system that ensures a safe practice of biological control, while remaining realistic and manageable for the stakeholders involved. Extensive delays as a result of the necessity to perform ERAs could multiply the cost of biological control programmes, leaving industries struggling and holding back the development of new products in Europe and elsewhere (Hunt et al. 2008). As the European Commission has not taken the initiative to develop regulation specifically targeting IBCAs, regulation of the import and release of IBCAs across Europe is fragmented (Bale 2011a). Each biological control company needs to submit an application dossier in every country where they wish to register their product, often with different information requirements. In order to harmonize the procedures in all EU countries, the REBECA project produced a standardized application form to be used by companies when applying for a licence to bring a non-native IBCA to the market. A revised version of the form was incorporated into EPPO’s Standard PM6 (“Safe Use of Biological Control”). It would be helpful if this form became the standard document to be used in all EU countries (Bale 2011a; Ehlers 2011). Regulating the use of exotic natural enemies across national borders, for example among different countries of a similar ecoregion in the EU, would thus result in a more harmonized and user-friendly system (Cock et al. 2006). In this way, a biocontrol company would have to submit only one dossier per ecoregion to obtain a permit for release (i.e. commercial distribution) for all countries within that ecoregion.
However, it remains unclear who will be responsible for evaluating the application dossiers, as in many cases specific expertise is lacking in the competent authorities. The establishment of an international panel of experts would be recommendable, but financial support for this initiative may not be easily found. If Europe is willing to accept this responsibility, establishing one expert panel that evaluates all application files for releases anywhere in Europe and that takes a decision for each ecoregion separately seems the most appropriate way to harmonize regulation of this matter. When the evaluation of application dossiers can be centralised, each biocontrol company still needs to register its own products, leading to several application files for the same natural enemy species. This procedure could be simplified by developing a system in which one company prepares the dossier for a certain natural enemy and other companies have the possibility to purchase the results of the research underlying the application dossier from that company. Such a system would benefit all parties as the company conducting the ERA has the opportunity to compensate for its research investments while other companies avoid these investment costs. Of course, the exchange of ERA results will only be possible when the involved companies are distributing the same biocontrol “product” as several factors related to the origin and rearing of the natural enemy under study may affect the outcome of the ERA.

Biological and ecological traits of a species of natural enemy may vary among different populations of that species (Messing and Rabasse 1995; Gotoh et al. 2004). For example, the biocontrol population of the harlequin ladybird *Harmonia axyridis* (Pallas)(Coleoptera: Coccinellidae) that became invasive in Europe has a negative impact on the native two-spotted ladybird *Adalia bipunctata* (L.)(Coleoptera: Coccinellidae)(Brown et al. 2011; Roy et al. 2012), whereas the *A. bipunctata* population introduced in Japan appears to be competing with and replacing native ladybird species, including *H. axyridis* (Toda and Sakuratani 2006). This is related to the particular genetic background of the invasive populations (Lombaert et al. 2011). Besides, biocontrol practitioners may be interested in populations of a given natural enemy that possesses traits making them more effective control agents. Non-diapausing populations of the predatory mite *Neoseiulus*
californicus (McGregor) (Acari: Phytoseiidae) are preferred over populations that undergo diapause under short day conditions as the first can be used more effectively during spring and autumn. This characteristic may affect the assessment of the establishment potential of N. californicus in the framework of an ERA (Jolly 2000; Hart 2002b). Therefore, Loomans and van Lenteren (2006) recommended to perform the ERA procedure for a candidate BCA at the population level, rather than at the species level. This view has been challenged, particularly by practitioners of biological control, who point at the practical complications this approach would generate, e.g. as related to difficulties to accurately identify and characterize a population (Ehlers 2011). In the light of simplifying the application procedure, it remains essential that the company conducting the ERA specifies the origin of the population under study and that any other company willing to use these results rears a population of the same origin. In practice, this condition is often fulfilled as many companies exchange their starting material for establishing a laboratory culture of a given natural enemy.

Besides the genetic structure of a population, our study shows that several factors related to the rearing of a natural enemy may also affect the outcome of an ERA. These include food sources used, climatic adaptations, and interactions with bacterial endosymbionts. For instance, the flight performance of predatory ladybirds fed the factitious food source Ephestia kuehniella Zeller (Lepidoptera: Pyralidae) eggs was superior to that of those reared on their natural prey, aphids (Chapter 7). Further, the freezing point of the predatory bug Macrolophus pygmaeus Rambur (Hemiptera: Miridae) was not only affected by diet, but also by low temperature acclimation and infection status with endosymbiotic Wolbachia and Rickettsia bacteria (Chapter 4). While bugs reared on an artificial diet were less resistant to freezing, short-term acclimation to low temperature and curing from bacterial infection resulted in an increase in cold tolerance. Also for the mealybug destroyer Cryptolaemus montrouzieri Mulsant (Coleoptera: Coccinellidae) a positive effect of the factitious diet and low temperature acclimation on the supercooling point was observed, but when assessing the impact of these factors on lethal time, a more realistic parameter of cold tolerance, no
significant effect could be detected (Chapter 6). Our findings suggest that in the light of an ERA, and in particular for sharing results among commercial biocontrol distributors, as well as in quality control protocols to be established at a later stage, a description of the rearing conditions of the BCA under study will be an essential requirement. While for a number of products that are commercially available rearing procedures are well-known, the situation is more complex for other, often newer products. In the latter case, companies have a competitive advantage by keeping their mass production protocol secret. On the other hand, patenting of rearing protocols is a relatively recent trend in commercial biological control and as registered patents are public, such an exchange of information on rearing procedures will occur anyway. The main question here is how detailed the information on rearing protocols has to be in the framework of an ERA procedure. Further research projects would be warranted to ascertain which other factors related to the mass rearing of natural enemies may affect ERA testing results. Testing the effects of rearing conditions on the ERA of more species of BCA will also help to further clarify this issue.

9.2 An environmental risk assessment for *C. montrouzieri*

Using *C. montrouzieri* as a case study, we have performed an ERA procedure applying the general guidelines that have been elaborated in the framework of the EU funded projects ERBIC and REBECA (van Lenteren et al. 2003; Ehlers 2011) and using the results of our testing protocols. We followed the flowchart developed by van Lenteren et al. (2006a), showing how to evaluate the different aspects of an ERA sequentially. In the first step, a distinction has to be made whether the BCA under study is either an indigenous or an exotic species. For *C. montrouzieri*, the outcome is straightforward as its original distribution is well described, reaching from eastern Australia (Queensland, New South Wales) to the South Pacific Islands (Clausen 1978; Booth and Pope 1986). Whereas the situation for *C. montrouzieri* is clear, our survey of commercially available BCAs indicated that information on the original distribution of other natural enemies is often incomplete or unclear (Chapter 3), which
complicates the continuation of the ERA procedure. When the BCA is considered native in the country where registration is requested, the ERA only consists of a study of possible (in)direct effects on non-target organisms. In contrast, the risk assessment for exotic BCAs is more extensive and may require a study of the BCA’s overwintering potential, prey range, dispersal capacity and potential (in)direct effects on non-targets. For an exotic natural enemy, the second step of the ERA will depend on the goal of the intended release (augmentation versus classical biological control) (van Lenteren et al. 2006a).

In northwestern Europe, the exotic ladybird *C. montrouzieri* is primarily used in augmentative biological control under protected cultivation. Because establishment outdoors is unwanted, an assessment of *C. montrouzieri*’s cold tolerance constitutes the second step of the ERA procedure (Chapter 6). The ladybird was found to be susceptible to chilling injury due to above-zero cold temperatures and when applying the relationship between LTime$_{50}$ at 5°C and field survival calculated by Hatherley et al. (2005) to our dataset, it could be predicted that *C. montrouzieri* would not persist in the field throughout western European winters. Therefore, the risk that the ladybird may establish in our regions can be considered small. The ERA procedure for release of *C. montrouzieri* in northwestern Europe can be terminated at this point and a permit for import and release can be granted (van Lenteren et al. 2006a). However, when winters in northwestern Europe become milder as a results of climate change, this decision should be reconsidered.

In southern Europe, *C. montrouzieri* may be able to survive winter, depending on the location. In this case, the study of its host or prey range constitutes the second step of an ERA (Loomans and van Lenteren 2005; van Lenteren et al. 2006a). Our experiments showed that the ladybird’s prey range is not limited to the Pseudococcidae, but includes other small, soft-bodied and sedentary hemipterans (Chapter 8). This non-specific feeding habit increases the risk that the predator will attack non-target species in agricultural and (semi-)natural habitats and affect their abundance. At this point in the risk assessment, there are 3 further options. The first option is perform additional testing on the
ladybird’s prey range before drawing firm conclusions about the ecological risks associated with the release of *C. montrouzieri*. The no-choice feeding tests like those conducted in the present study may overestimate the ladybird’s prey range, and further testing could be done conducting choice tests and/or experiments using more realistic arenas (e.g. caged plants) (van Driesche and Murray 2004b; Kuhlmann et al. 2006). Second, the risk that *C. montrouzieri* will threaten local biodiversity can be considered to be too extensive and the ERA procedure can be terminated here with a negative advice for introduction. Alternatively, the oligophagous feeding behaviour of *C. montrouzieri* can be recognised and resources can be invested into a study of the predator’s dispersal capacity as a the next step of an ERA. Even a polyphagous species can qualify for registration when its dispersal capacity is limited and potential side-effects are only expected to remain local (Ehlers 2011).

For the study of *C. montrouzieri*’s dispersal potential, we compared its flight capacity with the performance of the native ladybird *A. bipunctata* and the invasive ladybird *H. axyridis* in a computer-monitored flight mill (Chapter 7). The mealybug destroyer not only outperformed *A. bipunctata* but its total flight distance also equalled that of the invasive harlequin ladybird, *H. axyridis*, when both were fed their natural prey. Because the latter species is known to be a powerful flier (Obata 1986; Hodek et al. 1993; Tourniaire et al. 2000), we can conclude that the dispersal potential of *C. montrouzieri* is extensive and that the ladybird is capable of dispersing into the surrounding environment when the population of the target prey is locally depleted. At this point, further time could be invested in the study of the dispersal potential of *C. montrouzieri* by conducting mark-release-recapture experiments at the field scale (in areas where winter conditions would not allow the insects to survive), in order to validate the outcome of the flight mill tests (Mills et al. 2006). Flight mill experiments like those conducted in this study tend to overestimate the dispersal capacity of insects because the insects are forced to fly by lack of tarsal contact, but, on the other hand, insects flying under real conditions can be borne very far by the wind (Shirai and Kosugi 2000; Yamanaka et al. 2001; Blackmer et al. 2004; Botero-Garces and Isaacs 2004; Edwards 2006). Alternatively, the regulator can consider that the risk of a release is too high and choose to end the
ERA procedure with a negative advice for release. A final option is to assess the possible (in)direct effects of an introduction of *C. montrouzieri* on local biodiversity (van Lenteren et al. 2006a).

In this context, a number of laboratory studies pointed out that the mealybug destroyer might act as an intraguild predator and may dominate the coccidophagous guild. Chong and Oetting (2007) found that both ladybird larvae and adults display a similar preference for healthy mealybugs and mealybugs parasitized by the parasitoid *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae). Also Muştu et al. (2008) reported that *C. montrouzieri* does not discriminate between mealybugs parasitized or not by *Anagyrus pseudococci* (Girault)(Hymenoptera: Encyrtidae). Furthermore, in laboratory no-choice tests the ladybird fed on larvae of the predatory butterfly *Spalgis epius* (Westwood)(Lepidoptera: Lycaenidae), which could potentially undermine the biological control functions of the butterfly (Dinesh and Venkatesha 2009). Based on the laboratory experiments from our and previous studies, direct effects on non-target organisms cannot be excluded. Because these (mainly no-choice) tests in small Petri dishes may lead to an overestimation of the predation in the field, further testing may need to be done under more realistic conditions, in order to allow the making of a sound decision about an introduction of *C. montrouzieri* in areas where it can establish.

Based on the results of this study and information in the literature, an introduction of *C. montrouzieri* in southern Europe is not without risks. Especially its oligophagous feeding habit and potential effects on non-target organisms are of concern. A negative advice for import and release in areas where it can establish could therefore be in place. However, our and previous studies only provide fragmentary evidence and there may as yet not be sufficient information to support a decision in any direction. Furthermore, *C. montrouzieri* has a long history of introductions around the world and despite its intensive use in both classical and augmentative biological control there are no reports of invasiveness for this ladybird. Whereas on the one hand its oligophagous feeding habit increases the risks of undesired side-effects, oligophagy can also be an advantageous trait that may even result in positive effects on the local biodiversity. For example, after the introduction of *C. montrouzieri* in
Hawaii a field survey was conducted to evaluate its feeding ecology (Funasaki et al. 1988). The ladybird's field prey range included the target prey, 1 native mealybug species and 14 exotic pest species. Unless if *C. montrouzieri* would develop a preference for this native species over other prey species, which was deemed unlikely, the risk that the ladybird will cause the extinction of the native species was considered small. More importantly, the mealybug destroyer was expected to contribute to the conservation of local plant and animal biodiversity by feeding on exotic pest species. Therefore, when considering *C. montrouzieri* for release, the ultimate decision of regulators will strongly depend on the outcome of the risk and benefit analysis.

Cock (2013) summarized the risks of potential non-target impacts and stakeholder benefits related to the release of *C. montrouzieri* to control the hibiscus mealybug *M. hirsutus* in Grenada. It was concluded that political and social pressure demanded the introduction of the ladybird irrespective of the associated risks and a permit for release was granted. Meanwhile, the benefits of the introduction of the mealybug destroyer in Grenada are very clear and so far, there are no reports of undesired side-effects (Cock 2013). The *Cryptolaemus* case study nicely illustrates the complexity of predicting the impact of the release of an exotic natural enemy on local biodiversity based on laboratory experiments. Furthermore, this case study stresses the importance of the EPPO positive list. This list presents all BCAs used for at least 5 years in 5 EPPO countries without any reports of negative side-effects and thus provides historical evidence that can be taken into account by regulators when judging application dossiers for the release of BCAs. However, more efforts should be done to perform a retrospective analysis of previous biological control programs (i.e. by performing post-release studies), which would help to link the results of laboratory assessments to the actual impact of the released natural enemy in the field.
9.3 Improvements for *C. montrouzieri* rearing

In the margin of this study, significant improvements of the rearing procedures for *C. montrouzieri* were accomplished. We proved that *C. montrouzieri* females do not depend on mealybugs to trigger oviposition. The predator can be reared in the absence of mealybugs when eggs of the flour moth *E. kuehniella* are offered as a food source and synthetic wadding is provided as an oviposition substrate (Chapter 5). Lepidopteran eggs are a commonly used food for the mass production of predatory coccinellids, anthocorids, mirids and chrysopids (Morales-Ramos et al. 2014b). The good success obtained in several insect predators with these eggs could be related to their nutritional composition (Specty et al. 2003). Furthermore, flour moth eggs have good practical value in a production environment, as they are easily stored and dosed. Apparently, the polyester wadding successfully mimics the filamentous structure of mealybug ovisacs and in combination with a nutritionally adequate and easily handled food source the presence of mealybugs is no longer required to trigger oviposition in *C. montrouzieri* females. A disadvantage to the use of *E. kuehniella* eggs in a mass production system is their high cost due to investments for mechanization for rearing procedures, health care for workers and high costs for food supply and labour (Arijs and De Clercq 2001, De Clercq et al. 2014). Because the synthetic wadding is inexpensive, the amount of *E. kuehniella* eggs required to support development of *C. montrouzieri* will determine the cost-effectiveness of our semi-artificial rearing method. On average, ladybird larvae consumed each 45 mg of *E. kuehniella* eggs during their larval stages. With a current market price of 400 EUR/kg for flour moth eggs (K. Bolckmans, Koppert BV, pers. comm.), the production cost for one *C. montrouzieri* adult would be around 0.02 EUR, which should make the rearing system economically profitable (R. Timmer, Koppert BV, pers. comm.). Further research is warranted to optimize the presentation of the oviposition substrate. Females prefer to lay their eggs on the edges of wadding cuttings, which results in locally high densities of eggs and increases the risk of egg cannibalism when the first larvae hatch.
9.4 Conclusion

In conclusion, our study highlights the complexity of designing testing protocols to support the ERA for invertebrate natural enemies used in biological pest control. It has indicated the benefits and drawbacks of laboratory testing protocols for the different steps of an ERA and has demonstrated that several factors related to the production of the natural enemy may affect the outcome of an ERA. Whereas the implications of our study should be helpful for optimizing ERA procedures, more work is needed to develop a reliable and cost-effective methodology for the assessment of the risks of invertebrate predators, parasitoids and parasites used for the biological control of agricultural pests.
Summary

Both indigenous and exotic natural enemies are used in commercial biological control to reduce pest populations in agricultural crops. As exotic biological control agents (BCA) could have a negative impact on non-target organisms and may threaten local biodiversity, their use is not without risks. An appropriate regulation concerning the import and release of natural enemies is instrumental in preventing non-target effects (Chapter 2). As a first step to support regulatory initiatives in Belgium within the framework of the Macroreg project funded by the Federal Belgian authorities, invertebrate natural enemies that are currently commercially available to Belgian growers were inventoried and the proportion of exotic species out of the total assortment was estimated (Chapter 3). The survey indicated that currently more than 90 species of invertebrate natural enemies are commercially available to Belgian growers on the European market and that about 53% of these are exotic to Belgium or to the neighbouring countries within the same ecoregion. All continents were found to deliver more or less 20% of the exotic species (Asia: 17%, Africa: 20%, North America: 17%, Oceania: 17%, Europe: 24%), with the exception of South America that delivered only 5% of the exotic species. The European BCAs that have not previously been described from Belgium or surrounding areas mostly have a Mediterranean origin. We concluded that information on the original distribution of natural enemies is often incomplete or unclear and that questions can be raised about the feasibility of considering a small geographical area like Belgium or even Flanders (see "Soortenbesluit") as an ecologically relevant entity. The development of a harmonised regulation among different countries of a similar ecoregion is therefore recommendable.

A regulation concerning the use of exotic BCAs needs to be built on a scientifically sound environmental risk assessment (ERA). Two previous European initiatives, ERBIC (Evaluating Environmental Risks of Biological Control Introductions into Europe) and REBECA (Regulation of Biological Control Agents), have formulated general guidelines to perform ERA testing, integrating
information on a candidate BCA’s potential to establish, its abilities to disperse, its host range, and its
direct and indirect effects on non-target organisms. However, a concrete experimental methodology
to study the different aspects on an ERA is often still lacking. Cold tolerance studies are central to
assess the establishment potential of an exotic natural enemy and constitute a first step in the ERA
(Chapter 2). In Chapter 8, the cold tolerance of the Mediterranean biocontrol population of the
predatory bug *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) was assessed by determining
the supercooling point (SCP), i.e. the temperature at which the insect’s body fluids freeze. This
parameter provides a first indication of an insect’s establishment potential in a new region and of its
possible geographical range. Further, the influence of acclimation, infection with endosymbiotic
bacteria and diet on the SCP of *M. pygmaeus* was evaluated. Allowing the predatory bugs to adapt to
lower temperatures resulted in an increase in supercooling ability. *Macrolophus pygmaeus* bugs
exposed to antibiotics in their artificial diet and hence cured from their infection with the
endosymbiotic bacteria *Wolbachia pipiensis*, *Rickettsia bellii* and *R. limoniae* were more tolerant to
freezing than infected bugs. The diet of the predators also affected the freezing temperature of the
body fluids. Predators fed an artificial diet based on egg yolk were less resistant to freezing than
those fed eggs of the flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). These findings
illustrate that several factors may affect the cold hardiness of a BCA population and may thus
complicate the evaluation of its establishment potential in the framework of an ERA.

The influence of rearing conditions on the outcome of an ERA testing procedure was further
investigated using the mealybug destroyer *Cryptolaemus montrouzieri* Mulsant (Coleoptera:
Coccinellidae) as a case study. In a first step, a semi-artificial rearing for the Australian ladybird was
developed (Chapter 4). Our rearing system, using *E. kuehniella* eggs as a food and synthetic polyester
wadding as an oviposition substrate, was compared with a natural rearing system, using the citrus
mealybug *Planococcus citri* (Risso)(Hemiptera: Pseudococcidae), as to its effects on the predator’s
developmental and reproductive parameters. In a second series of experiments the performance of
*C. montrouzieri* on bee pollen or on a mixture of *E. kuehniella* eggs and bee pollen was assessed.
Flour moth eggs proved to be a suitable food to support larval development of the predator. Ladybird larvae reared on flour moth eggs developed 2 days faster and weighed approximately 10% more than their counterparts reared on mealybugs. Despite a prolongation of the preoviposition period with ca. 8 days and a decrease in egg hatch by about 10%, *C. montrouzieri* females fed moth eggs accepted the synthetic wadding as an oviposition substrate and deposited similar numbers of eggs as their counterparts maintained on mealybugs. A mixture of *E. kuehniella* eggs with pollen yielded similar developmental and reproductive rates as *E. kuehniella* eggs alone, but a diet of bee pollen alone was not adequate for the predator. These experiments indicate the potential of a rearing system based on *E. kuehniella* eggs as a factitious food and synthetic wadding as an artificial oviposition substrate for the mass production of *C. montrouzieri*.

In a second step, the cold tolerance and dispersal potential of naturally reared *C. montrouzieri* ladybirds was compared to the overwintering and dispersal capacity of ladybirds reared according to the semi-artificial rearing method. Chapter 5 addressed the effect of diet on the SCP and lethal time (i.e. time required to kill 50% of the population at a temperature of 5°C) of *C. montrouzieri*. Further, the influence of low temperature acclimation on the parameters of cold tolerance was studied. The SCP of acclimated adult ladybirds which were allowed to complete development at 18°C and a 8:16(L:D)h photoperiod, or at 25°C and a 16:8(L:D)h photoperiod, averaged -17.4°C and -16.8°C, respectively, and was approximately 7°C lower than the value of -9.9°C for ladybirds maintained at a temperature of 25°C and a photoperiod of 16:8(L:D)h. Also food source had a significant effect on the freezing temperature of *C. montrouzieri*: the SCP of ladybirds fed *P. citri* mealybugs was 1.6°C higher than the value of -17.2°C observed for ladybirds provided with *E. kuehniella* eggs. However, neither cold acclimation nor diet had a significant effect on the lethal times of *C. montrouzieri*. Overall, the time required to kill 50% of the population at a temperature of 5°C ranged from 12.8 days for ladybirds fed *P. citri* mealybugs to 14.4 days for ladybirds fed *E. kuehniella* eggs. All individuals exposed to a constant 5°C had died by day 24. Based on the results from this laboratory study, it is deemed unlikely that *C. montrouzieri* could establish outdoors in western Europe, and the coccinellid...
is therefore expected to pose little risk to non-target species in this area when used as an augmentative BCA. These experiments again indicate the complexity of predicting the cold hardiness of a candidate BCA, more specifically as to which factors should be taken into consideration when standardizing an experimental protocol for assessing its establishment potential in the framework of an ERA.

In **Chapter 6**, a computer-monitored flight mill was proposed as a tool in ERA procedures to assess the dispersal capacity of predatory ladybirds. The flight potential of *C. montrouzieri* was compared with the performances of the native two-spotted ladybird *Adalia bipunctata* (L.)(Coleoptera: Coccinellidae) and the invasive harlequin ladybird *Harmonia axyridis* (Pallas)(Coleoptera: Coccinellidae). Further, the effect of diet on the flight parameters of the three coccinellids was tested. Overall, ladybirds reared on eggs of *E. kuehniella* performed better than their counterparts reared on natural prey (aphids for *H. axyridis* and *A. bipunctata*, mealybugs for *C. montrouzieri*). *Harmonia axyridis* flew at least 2 times further, needed 3 times less breaks and flew 2 times faster than *A. bipunctata* fed the same diet. Also the mealybug destroyer outperformed *A. bipunctata* but its total flight distance equalled that of *H. axyridis* when both were fed their natural prey. Because *H. axyridis* is known to be a powerful flier, we concluded that the dispersal potential of *C. montrouzieri* is extensive and that the ladybird is capable of dispersing into the surrounding environment when the population of the target prey is locally depleted. These experiments showed that comparative flight studies can be useful to identify candidate BCAs with pronounced dispersal abilities and thus can yield significant evidence to be used in an ERA procedure. However, it also demonstrates that variability related to mass rearing conditions should not be ignored when standardizing a risk assessment test protocol for candidate BCAs.

As a last aspect of the ERA procedure for *C. montrouzieri*, its prey range was studied in the laboratory (**Chapter 7**). Prey tested in these experiments were: tobacco aphid *Myzus persicae nicotianae* (Sulzer)(Hemiptera: Aphididae), pea aphid *Acyrthosiphon pisum* (Harris)(Hemiptera: Aphididae),
tobacco whitefly *Bemisia tabaci* (Gennadius)(Hemiptera: Aleurodidae), southern green stinkbug *Nezara viridula* (L.)(Hemiptera: Pentatomidae), western flower thrips *Frankliniella occidentalis* (Pergande)(Thysanoptera: Thripidae), two-spotted ladybird *Adalia bipunctata* (L.)(Coleoptera: Coccinellidae), yellow mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae). Larval survival was high to moderate when *C. montrouzieri* was provided with hemipteran prey and poor to zero when the ladybirds were provided with the tested non-hemipteran prey. Females reared on *M. persicae* and *A. pisum* produced similar numbers of eggs as their counterparts fed mealybugs, but fecundity was strikingly lower when the ladybirds were reared on *B. tabaci* nymphs or on *A. bipunctata* eggs. Prey species that were found to be less suitable for immature development of *C. montrouzieri* could still be an adequate food source for reproduction and survival of adult ladybirds. For example, only 8% of the predator larvae reached the adult stage when provided with *A. bipunctata* eggs, but females that had developed on *E. kuehniella* eggs and that were supplied with *A. bipunctata* eggs from adult emergence on, were only 35% less fecund than females provided with mealybugs in their adult life. Our experiments showed that the ladybird’s prey range is not limited to the Pseudococcidae, but includes other small, soft-bodied and sedentary hemipterans. This non-specific feeding habit increases the risk that the predator will attack non-target species in agricultural and (semi-)natural habitats and affect their abundance. However, no-choice laboratory tests may overestimate this risk and before drawing firm conclusions about the ecological risks associated with the release of *C. montrouzieri* in areas where it may establish, further testing should be done by performing choice tests and/or experiments using more realistic arenas (e.g. caged plants).

In Chapter 9, a general discussion of the findings is presented and future prospects are discussed. It is concluded that designing testing protocols to support the ERA for invertebrate natural enemies used in biological pest control remains a complicated exercise as several factors related to the rearing of the natural enemy may affect the outcome of an ERA test protocol. Whereas the implications of our study should be helpful for optimizing ERA procedures, more work is needed to
develop a reliable and cost-effective methodology for the assessment of the risks of invertebrate predators, parasitoids and parasites used for the biological control of agricultural pests.
In de commerciële biologische gewasbescherming worden zowel inheemse als uitheemse natuurlijke vijanden uitgezet om plaaginsecten te bestrijden in land- en tuinbouwgewassen. Het gebruik van exotische biologische bestrijders is echter niet zonder risico aangezien zij een negatieve impact kunnen uitoefenen op niet-doelorganismen en de locale biodiversiteit kunnen bedreigen. Een geschikte regulering omtrent de import en het uitzetten van natuurlijke vijanden is noodzakelijk om ongewenste neveneffecten te voorkomen (Hoofdstuk 2). Als een eerste stap om de ontwikkeling van een mogelijke wetgeving in België rond dit thema te ondersteunen werden, in het kader van een project gefinancierd door de Belgische federale overheid (Macroreg), de invertebrate natuurlijke vijanden geïnventariseerd die momenteel commercieel beschikbaar zijn op de Europese markt en dus ook voor Belgische land- en tuinbouwers. Bovendien werd ingeschat wat het aandeel is van exotische soorten uit het totale assortiment (Hoofdstuk 3). De inventaris toonde aan dat momenteel meer dan 90 verschillende soorten natuurlijke vijanden beschikbaar zijn op de Europese markt en dat 53% van deze soorten exotisch zijn voor België of de naburige landen behorende tot dezelfde ecoregio. Alle continenten bleken ongeveer 20% van deze exotische soorten aan te leveren (Azië: 17%, Afrika: 20%, Noord-Amerika: 17%, Oceanië: 17%, Europa: 24%), met als uitzondering Zuid-Amerika dat slechts in 5% van de exoten voorzag. De Europese biologische bestrijders die niet eerder werden beschreven voor België of omliggende streken hadden meestal een mediterrane oorsprong. We concludeerden dat informatie omtrent de herkomst van natuurlijke vijanden vaak onvolledig of onduidelijk is en dat vragen kunnen gesteld worden bij het beschouwen van kleine geografische gebieden zoals België of Vlaanderen (zie ‘Soortenbesluit’) als een ecologisch relevante entiteit. De ontwikkeling van een geharmoniseerde wetgeving tussen verschillende landen van eenzelfde ecoregio is daarom aangewezen.

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In de commerciële biologische gewasbescherming worden zowel inheemse als uitheemse natuurlijke vijanden uitgezet om plaaginsecten te bestrijden in land- en tuinbouwgewassen. Het gebruik van exotische biologische bestrijders is echter niet zonder risico aangezien zij een negatieve impact kunnen uitoefenen op niet-doelorganismen en de locale biodiversiteit kunnen bedreigen. Een geschikte regulering omtrent de import en het uitzetten van natuurlijke vijanden is noodzakelijk om ongewenste neveneffecten te voorkomen (Hoofdstuk 2). Als een eerste stap om de ontwikkeling van een mogelijke wetgeving in België rond dit thema te ondersteunen werden, in het kader van een project gefinancierd door de Belgische federale overheid (Macroreg), de invertebrate natuurlijke vijanden geïnventariseerd die momenteel commercieel beschikbaar zijn op de Europese markt en dus ook voor Belgische land- en tuinbouwers. Bovendien werd ingeschat wat het aandeel is van exotische soorten uit het totale assortiment (Hoofdstuk 3). De inventaris toonde aan dat momenteel meer dan 90 verschillende soorten natuurlijke vijanden beschikbaar zijn op de Europese markt en dat 53% van deze soorten exotisch zijn voor België of de naburige landen behorende tot dezelfde ecoregio. Alle continenten bleken ongeveer 20% van deze exotische soorten aan te leveren (Azië: 17%, Afrika: 20%, Noord-Amerika: 17%, Oceanië: 17%, Europa: 24%), met als uitzondering Zuid-Amerika dat slechts in 5% van de exoten voorzag. De Europese biologische bestrijders die niet eerder werden beschreven voor België of omliggende streken hadden meestal een mediterrane oorsprong. We concludeerden dat informatie omtrent de herkomst van natuurlijke vijanden vaak onvolledig of onduidelijk is en dat vragen kunnen gesteld worden bij het beschouwen van kleine geografische gebieden zoals België of Vlaanderen (zie ‘Soortenbesluit’) als een ecologisch relevante entiteit. De ontwikkeling van een geharmoniseerde wetgeving tussen verschillende landen van eenzelfde ecoregio is daarom aangewezen.
Een reguleringsregeling omtrent het gebruik van exotische biologische bestrijders dient te steunen op een wetenschappelijk onderbouwde risico-`inschatting. Twee voormalige Europese initiatieven, ERBIC (Evaluating Environmental Risks of Biological Control Introductions into Europe) en REBECA (Regulation of Biological Control Agents), hebben algemene richtlijnen geformuleerd om een risico-`inschatting uit te voeren. Een belangrijke conclusie van deze initiatieven was dat een risico-`inschatting gebaseerd moet zijn op het vestigingsvermogen, het verspreidingsvermogen en het prooi`bereik van de kandidaat biologische bestrijder en de mogelijke directe en indirecte effecten die de natuurlijke vijand zou kunnen veroorzaken op niet-doelorganismen. Een concrete, experimentele methodologie om de verschillende aspecten van de risico-`inschatting te bestuderen is echter vaak niet voorhanden. Enkel voor de studie van het vestigingsvermogen van een exotische natuurlijke vijand werd eerder een concrete methodiek uitgewerkt, steunend op de koudetolerantie van de exoot (Hoofdstuk 2). In Hoofdstuk 8 bestudeerden we het overwinteringvermogen van de mediterrane populatie van de roofwants *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae), die beschikbaar is voor biologische bestijding in onze kassen, aan de hand van het onderkoelingspunt. Dit is de temperatuur waarop de lichaamsvloeistoffen van het insect bevriezen. Deze parameter geeft een eerste indicatie over het vestigingsvermogen in een nieuwe regio en de potentiële geografische verspreiding van het insect. Bijkomend werd ook het effect van acclimatisatie, infectiestatus met endosymbionten en dieet op het onderkoelingspunt van *M. pygmaeus* geëvalueerd. De roofwantsen geleidelijk laten aanpassen aan lage temperaturen resulteerde in een toename van het onderkoelingvermogen. Predators die antibiotica aangeboden kregen in hun artificiële dieet en die bijgevolg niet langer geïnfecteerd waren met de endosymbionten *Wolbachia pipientis*, *Rickettsia bellii* en *R. limoniae* bleken meer tolerant voor bevriezing dan geïnfecteerde roofwantsen. Het dieet dat de roofwantsen aangeboden kregen had ook een effect op de bevriezingstemperatuur. Wantsen gevoed met een artificieel dieet op basis van eigeel waren minder bestendig tegen bevriezing dan deze gevoed op eitjes van de meelmot *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae). Deze bevindingen illustreren dat verschillende factoren de koudetolerantie van een populatie van een
biologische bestrijder kunnen beïnvloeden en dat deze de evaluatie van zijn vestigingspotentieel in het kader van een risico-inschatting bemoeilijken.

De invloed van kweekomstandigheden op de uitkomst van een risico-inschattingsprocedure werd verder bestudeerd met de wolluisbestrijder Cryptolaemus montrouzieri Mulsant (Coleoptera: Coccinellidae) als case study. In een eerste fase werd een semi-artificieel kweeksystem voor het Australisch lieveheersbeestje ontwikkeld (Hoofdstuk 4). Onze kweekmethode, gebruik makend van E. kuehniella eitjes als voedingsbron en synthetische watten als ovipositieondergrond, werd vergeleken met het natuurlijke kweeksystem, dat gebaseerd is op de citruswolluis Planococcus citri (Risso)(Hemiptera: Pseudococcidae), aan de hand van de ontwikkeling- en reproductieparameters van C. montrouzieri. In een tweede reeks van experimenten werd de prestatie van het lieveheersbeestje op bijenpollen en een mengsel van bijenpollen en E. kuehniella eitjes nagegaan. Meelmoteitjes bleken een geschikte voeding om de larvale ontwikkeling van de predator te ondersteunen. Larven van het lieveheersbeestje gekweekt op deze eitjes ontwikkelden 2 dagen sneller en wogen ongeveer 10% meer dan larven gevoed met wolluizen. Ondanks een toename in preovipositieperiode van ongeveer 8 dagen en een afname in het ontluikingspercentage van C. montrouzieri eitjes met ongeveer 10%, accepteerden de C. montrouzieri wijfjes gevoed met meelmoteitjes het artificiële ovipositieondergrond en legden een vergelijkbaar aantal eitjes in vergelijking met vrouwtjes gekweekt op wolluizen. Het mengsel van meelmoteitjes en bijenpollen resulteerde in gelijkaardige ontwikkeling- en reproductieparameters als zuivere E. kuehniella, maar een dieet op bijenpollen alleen bleek geen geschikte voedingsbron voor de predator. Deze experimenten tonen het potentieel aan van een kweekmethode gebaseerd op meelmoteitjes als onnatuurlijke prooi en synthetische watten als artificieel ovipositieondergrond voor de massakweek van C. montrouzieri.

In een tweede fase werd de koudetolerantie en het verspreidingsvermogen van natuurlijk gekweekte C. montrouzieri lieveheersbeestjes vergeleken met het overwintering- en verspreidingsvermogen van
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lieveheersbeestjes gekweekt volgens het semi-artificiële kweekssysteem. **Hoofdstuk 5** behandelt het effect van dieet op het onderkoelingspunt en de letale tijd (tijd alvorens 50% van de populatie afgedood wordt op een temperatuur van 5°C) van *C. montrouzieri*. Verder werd de invloed van acclimatisatie op deze parameters van koudetolerantie bestudeerd. Het onderkoelingspunt van geacclimatiseerde volwassen lieveheersbeestjes die hun ontwikkeling volbrachten bij een temperatuur van 18°C en een fotoperiode van 8:16(L:D)u of een temperatuur van 25°C en een fotoperiode van 16:8(L:D)u bedroeg respectievelijk -17.4°C en -16.8°C. Dit was ongeveer 7°C lager dan de waarde van -9.9°C geobserveerd voor lieveheersbeestjes opgekweekt op een temperatuur van 25°C en een fotoperiode van 16:8(L:D)u. Ook de voedingsbron had een significant effect op het onderkoelingspunt van *C. montrouzieri*: de bevriezingsstemperatuur van lieveheersbeestjes gevoed op *P. citri* wolluizen was 1.6°C hoger dan de waarde van -17.2°C geobserveerd voor lieveheersbeestjes voorzien van *E. kuehniella* eitjes. Noch acclimatisatie noch dieet hadden echter een significant effect op de letale tijd van *C. montrouzieri*. Algemeen schommelde de tijd vereist om 50% van de populatie af te doden bij 5°C van 12.8 dagen voor lieveheersbeestjes gevoed op *P. citri* wolluizen tot 14.4 dagen voor lieveheersbeestjes voorzien van meelmoteitjes. Alle individuen blootgesteld aan een constante temperatuur van 5°C stierven binnen de 24 dagen. Gebaseerd op de resultaten van deze laboratoriumstudie, wordt het onwaarschijnlijk geacht dat *C. montrouzieri* zich zou kunnen vestigen in West-Europa en het risico dat dit lieveheersbeestje neveneffecten zal teweeg brengen op niet-doelorganismen kan dan ook als laag beschouwd worden. Deze experimenten tonen opnieuw de complexiteit aan in het voorspellen van de koudetolerantie van een kandidaat biologische bestrijder. Meer specifiek, welke factoren in overweging genomen moeten worden tijdens het standaardiseren van testprotocollen voor de studie van het overwinteringvermogen in het kader van een risicoreductie.

In **Hoofdstuk 6** werd de "flight mill" voorgesteld als een middel om het verspreidingsvermogen van roofkevers in kaart te brengen. Het vliegpotentieel van *C. montrouzieri* werd hiertoe vergeleken met de prestaties van het inheemse, tweestippelige lieveheersbeestje *Adalia bipunctata* (L.) (Coleoptera:
Coccinellidae) en het invasieve, veelkleurige lieveheersbeestje *Harmonia axyridis* (Pallas)(Coleoptera: Coccinellidae). Bijkomend werd het effect van dieet op de vliegparameters van deze drie roofkevers nagegaan. Algemeen presteerden lieveheersbeestjes gevoed op *E. kuehniella* eitjes beter dan de kevers gevoed op hun natuurlijke prooi (bladluizen voor *H. axyridis* en *A. bipunctata*, wolluizen voor *C. montrouzieri*). *Harmonia axyridis* vloog minstens 2 keer verder, had 3 keer minder rustpauzes nodig en vloog 2 keer sneller dan *A. bipunctata* gevoed op hetzelfde dieet. Ook het Australisch lieveheersbeestje presteerde beter dan *A. bipunctata*, maar had eenzelfde vliegafstand als *H. axyridis* wanneer beide kevers gevoed werden op hun natuurlijke prooi. Aangezien *H. axyridis* gekend staat als een krachtige vlieger, besloten we dat het verspreidingsvermogen van *C. montrouzieri* aanzienlijk is en dat dit lieveheersbeestje in staat is om zich te verspreiden in de omgeving wanneer de populatie van het doelorganisme locaal uitgeput is. Deze proeven toonden aan dat vergelijkende "flight mill" studies nuttig kunnen zijn om kandidaat biologische bestrijders met uitgesproken verspreidingsmogelijkheden te identificeren en dat zulke studies bruikbaar bewijsmateriaal kunnen opleveren voor een risico-inschattingsprocedure. Dit onderzoek toont echter ook aan dat variabiliteit gerelateerd aan kweekomstandigheden niet genegeerd kan worden bij het opstellen van testprotocollen voor kandidaat biologische bestrijders.

Als een laatste aspect van de risico-inschatting van *C. montrouzieri*, werd het prooibereik van dit lieveheersbeestje bestudeerd in het laboratorium *(Hoofdstuk 7)*. De geteste prooien in deze experimenten waren: tabaksluis *Myzus persicae nicotianae* (Sulzer)(Hemiptera: Aphididae), bonenluis *Acyrthosiphon pisum* (Harris)(Hemiptera: Aphididae), tabakswittevlieg *Bemisia tabaci* (Gennadius)(Hemiptera: Aleyrodidae), zuidelijke groene stinkwants *Nezara viridula* (L.)(Hemiptera: Pentatomidae), Californische trips *Frankliniella occidentalis* (Pergande)(Thysanoptera: Thripidae), twee- of doppeltje lieveheersbeestje *A. bipunctata*, zwarte meeltoor *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) en grote wasmot *Galleria mellonella* L. (Lepidoptera: Pyralidae). De larvale overleving was hoog tot gemiddeld wanneer *C. montrouzieri* gevoed werd met prooien behorende tot de orde van de Hemiptera en laag tot onbetaald wanneer de lieveheersbeestjes voorzien werden van de
testprooien behorende tot andere insectenordes. Vrouwtjes gevoed op *M. persicae* en *A. pisum* produceerden een gelijk aantal eitjes als wijfjes gevoed op wolluizen, maar de fecunditeit was opvallend lager wanneer de lieveheersbeestjes gekweekt werden op *B. tabaci* nimfen en *A. bipunctata* eitjes. Prooien die minder geschikt bevonden werden voor de larvale ontwikkeling van *C. montrouzieri* konden wel een geschikte dieet vormen voor de reproductie en overleving van adulte kevers. Bijvoorbeeld, slechts 8% van de larven bereikte het volwassen stadium wanneer ze voorzien werden van *A. bipunctata* eitjes, maar wijfjes die ontwikkelden op *E. kuehniella* eitjes en voorzien werden van *A. bipunctata* eitjes tijdens het adulte stadium, bleken maar 35% minder vruchtbaar dan vrouwtjes voorzien met wolluizen tijdens hun volwassen leven. Onze experimenten toonden aan dat het prooibereik van *C. montrouzieri* niet beperkt is tot de Pseudococcidae, maar ook andere kleine, weke en sedentaire Hemiptera beslaat. Dit niet-specifiek voedingsgedrag verhoogt het risico dat deze predator niet-doelorganismen zal aanvallen in regio’s waar hij zich kan vestigen. Niet-keuze experimenten zoals deze die hier uitgevoerd werden, kunnen echter dit risico overschatten. Alvorens sterke conclusies te trekken over de ecologische risico’s die verbonden zijn aan de uitzetting van *C. montrouzieri* in gebieden waar het lieveheersbeestje zich kan vestigen is verder onderzoek nodig en dienen keuze testen en/of experimenten in meer realistische kooien (met planten) uitgevoerd te worden.

Tot slot volgde in Hoofdstuk 9 een algemene discussie van onze bevindingen en werden toekomstperspectieven naar voor geschoven. Er werd geconcludeerd dat het ontwerpen van testprotocollen voor de risico-inschatting van invertebrate biologische bestrijders een gecompliceerde oefening blijft aangezien verschillende factoren gerelateerd aan de kweek van de natuurlijke vijand de uitkomst van het testprotocol kunnen beïnvloeden. Terwijl de implicaties van onze studie nuttig zijn om de risico-inschattingprocedure te optimaliseren, is er meer werk nodig om een betrouwbare en kostenefficiënte methodiek te ontwikkelen voor de risico-inschatting van invertebrate predators, parasitoiden en parasieten gebruikt voor de biologische bestrijding van plaaginsecten.
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**Appendix 1:** List of invertebrate natural enemies commercially available in 2012 to Belgian growers, their original distribution (based on information in EPPO standard PM6/3), their current distribution in Belgium and neighbouring countries (The Netherlands (N), Germany (G) and France (F); based on the database of Fauna Europaea), their status as exotic species in Belgium and neighbouring countries (based on the database of DAISIE) and whether they were considered native or exotic in our inventory.

<table>
<thead>
<tr>
<th>Biological control agents</th>
<th>Original distribution (EPPO*)</th>
<th>Current distribution (Fauna Europaea**)</th>
<th>Status as exotic species (DAISIE***</th>
<th>Inventory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Belgium</td>
<td>Neighbouring countries</td>
<td>Belgium</td>
<td>Neighbouring countries</td>
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<td><strong>INSECTA</strong></td>
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<tr>
<td><strong>Coleoptera</strong></td>
<td></td>
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<tr>
<td><em>Adalia bipunctata</em> (L.)</td>
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<td>Present (N, G, F)</td>
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<tr>
<td><em>Atheta coriaria</em> (Kraatz)</td>
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<td>-</td>
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<td><em>Chilocorus circumdatus</em> Gyllenhal</td>
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<td><em>Chilocorus nigritus</em> (Fabricius)</td>
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<td><em>Clitostethus arcuatus</em> (Rossi)</td>
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<td><strong>Coccinella septempunctata</strong> L.</td>
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<td>Present (N, G, F)</td>
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<td><em>Cryptolaemus montrouzieri</em> Mulsant</td>
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<td><em>Exochomus laeviusculus</em> Weise</td>
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<tr>
<td><em>Exochomus quadripustulatus</em> L.</td>
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<td>-</td>
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<td>-</td>
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<td><em>Harmonia axyridis</em> Pallas (flightless strain)</td>
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<td><em>Nephus includens</em> (Boheman)</td>
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<td><strong>Diptera</strong></td>
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<td><em>Feltiella acarisuga</em> (Vallot)</td>
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<td>Current distribution (Fauna Europaea**)</td>
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<td>Current distribution (Fauna Europaea**)</td>
<td>Status as exotic species (DAISIE***)</td>
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<td><strong>Encarsia hispida</strong> De Santis</td>
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<td>Established</td>
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<td><strong>Hungariella peregrina</strong> Compere</td>
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<tr>
<td><strong>Leptomastix abnormis</strong> (Girault)</td>
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<td><strong>Leptomastix dactylopii</strong> (Howard)</td>
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</tr>
<tr>
<td><strong>Lysiphlebus testaceipes</strong> (Cresson)</td>
<td>Nearctic</td>
<td>-</td>
<td>Present (F)</td>
<td>Established (F)</td>
</tr>
<tr>
<td><strong>Metaphycus flavus</strong> (Howard)</td>
<td>Nearctic</td>
<td>Absent</td>
<td>Present (F)</td>
<td>Established (F)</td>
</tr>
<tr>
<td><strong>Metaphycus helvolus</strong> (Compere)</td>
<td>South Africa</td>
<td>Absent</td>
<td>Present (F, G)</td>
<td>Established</td>
</tr>
<tr>
<td><strong>Metaphycus lounsburyi</strong> (Howard)</td>
<td>California, Australia, Hawaii, South Africa</td>
<td>-</td>
<td>-</td>
<td>Established (N, F)</td>
</tr>
<tr>
<td><strong>Microterys flavus</strong> (Howard)</td>
<td>California, Pakistan</td>
<td>Present</td>
<td>Present (N, G, F)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Opius pallipes</strong> Wesmael</td>
<td>Palearctic (?)</td>
<td>Present</td>
<td>Present (N, G, F)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Praon volucre</strong> (Haliday)</td>
<td>Palearctic</td>
<td>Present</td>
<td>Present (N, G, F)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Pseudaphycus maculipennis</strong> (Mercet)</td>
<td>Palearctic</td>
<td>Present</td>
<td>Present (F)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Scutellista cyanea</strong> Motsch</td>
<td>Africa</td>
<td>Absent</td>
<td>Present (F)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Trichogramma brassicae</strong> Bezdenko</td>
<td>Europe</td>
<td>Present</td>
<td>Present (N, G, F)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Trichogramma cacaeciae</strong> (Marchal)</td>
<td>Europe</td>
<td>-</td>
<td>Present (N, G, F)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Trichogramma evanescens</strong> Westwood</td>
<td>Europe</td>
<td>Present</td>
<td>Present (N, G, F)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Thripobius semiluteus</strong> Boucek</td>
<td>-</td>
<td>Absent</td>
<td>Absent (N, G, F)</td>
<td>-</td>
</tr>
</tbody>
</table>

**Neuroptera**

| Chrysoperla carnea** (Say) | Cosmopolitan | Present | Present (N, G, F) | - | Native |

**Thysanoptera**

<p>| Aleurodithrips fasciapennis** (Franklin) | - | - | - | Established | Established (G) | Exotic |
| Franklinthrips megalops** Trybom | Africa, Israel, India | - | - | - | Alien (N) | Exotic |
| Franklinthrips vespiformis** (Crawford) | Asia | - | - | Alien | Alien (N, F, G) | Exotic |</p>
<table>
<thead>
<tr>
<th>Biological control agents</th>
<th>Original distribution (EPPO*)</th>
<th>Current distribution (Fauna Europaea**)</th>
<th>Status as exotic species (DAISIE***</th>
<th>Inventory</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ARACHNIDA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Acarina</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Amblyseius andersoni</em> Chant</td>
<td>Palaeartic and Nearctic</td>
<td>-</td>
<td>-</td>
<td>Native</td>
</tr>
<tr>
<td><em>Amblyseius barkeri</em> (Hughes)</td>
<td>Europe</td>
<td>-</td>
<td>-</td>
<td>Native</td>
</tr>
<tr>
<td><em>Amblyseius californicus</em> McGregor</td>
<td>Southern North America, Mediterranean</td>
<td>-</td>
<td>-</td>
<td>Exotic</td>
</tr>
<tr>
<td><em>Amblyseius cucumeris</em> (Oudemans)</td>
<td>Cosmopolitan</td>
<td>-</td>
<td>-</td>
<td>Native</td>
</tr>
<tr>
<td><em>Amblyseius degenerans</em> Berlese</td>
<td>Africa, Mediterranean</td>
<td>-</td>
<td>-</td>
<td>Exotic</td>
</tr>
<tr>
<td><em>Amblyseius montdorensis</em> (Schicha)</td>
<td>Australia, Pacific islands</td>
<td>-</td>
<td>-</td>
<td>Exotic</td>
</tr>
<tr>
<td><em>Amblyseius swirskii</em> (Athias-Henriot)</td>
<td>East of Mediterranean region</td>
<td>-</td>
<td>-</td>
<td>Exotic</td>
</tr>
<tr>
<td><em>Hypoaspis aculeifer</em> (Canestrini)</td>
<td>Europe</td>
<td>-</td>
<td>-</td>
<td>Native</td>
</tr>
<tr>
<td><em>Hypoaspis miles</em> (Berlese)</td>
<td>Palaeartic</td>
<td>-</td>
<td>-</td>
<td>Native</td>
</tr>
<tr>
<td><em>Metaseiulus occidentalis</em> (Nesbitt)</td>
<td>Nearctic</td>
<td>-</td>
<td>-</td>
<td>Exotic</td>
</tr>
<tr>
<td><em>Phytoseiulus longipes</em> Evans</td>
<td>South Africa, Zimbabwe</td>
<td>-</td>
<td>-</td>
<td>Exotic</td>
</tr>
<tr>
<td><em>Phytoseiulus persimilis</em> Athias-Henriot</td>
<td>Mediterranean</td>
<td>-</td>
<td>-</td>
<td>Exotic</td>
</tr>
<tr>
<td><em>Typhlodromus pyri</em> Scheuten</td>
<td>Europe, Nearctic</td>
<td>-</td>
<td>-</td>
<td>Native</td>
</tr>
<tr>
<td><strong>NEMATODA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Heterorhabditis bacteriophora</em> (Poinar)</td>
<td>South and central Europe/North America</td>
<td>-</td>
<td>Present (N, G, F)</td>
<td>Native</td>
</tr>
<tr>
<td><em>Heterorhabditis megidis</em> (Poinar)</td>
<td>Europe</td>
<td>-</td>
<td>Present (N, G, F)</td>
<td>Native</td>
</tr>
<tr>
<td><em>Phasmarhabditis hermaphrodita</em> Schneider</td>
<td>Central Europe</td>
<td>-</td>
<td>Present (G, F)</td>
<td>Native</td>
</tr>
<tr>
<td><em>Steinernema carpocapsae</em> (Filipjev)</td>
<td>Europe (Holarctic)</td>
<td>-</td>
<td>Present (G, F)</td>
<td>Native</td>
</tr>
<tr>
<td><em>Steinernema feltiae</em> (Filipjev)</td>
<td>Europe (Holarctic)</td>
<td>Present</td>
<td>Present (N, G, F)</td>
<td>Native</td>
</tr>
<tr>
<td><em>Steinernema kraussei</em> (Steiner)</td>
<td>Europe, North America</td>
<td>Present</td>
<td>Present (N, G)</td>
<td>Native</td>
</tr>
</tbody>
</table>

*: '-' : no information available

**: '-' : no information available for this species in these specific countries; ‘present’: at least one well documented record since year 1600; ‘absent’: species not seen since year 1600 despite search in the area; ‘doubtful’: the source is doubtful (reference to a work known not to be at a correct scientific level), possible misidentification, uncertainty of the locality report, uncertainty of the origin of the animal (possible but not verified escape from a zoo, exotic pet abandoned by people, ...), species not seen since the year 1600 but not specifically searched in the area

**: '-' : no information available for this species in these specific countries; ‘established’: the species has formed self-reproducing populations where introduced; ‘alien’: a species introduced outside its natural past or present distribution; ‘cryptogenic’: widespread species not demonstrably indigenous or adventitious in the area
Curriculum vitae

Personalia

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**Publications**


Oral Presentation


Poster Presentation