



**BIOLOGICAL CONTROL OF *GONIPTERUS PLATENSIS*:
CURRENT STATUS AND NEW POSSIBILITIES**

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ORIENTADORA: Doutora Manuela Rodrigues Branco Simões

TESE ELABORADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA
FLORESTAL E DOS RECURSOS NATURAIS

2018

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2018

À Susana e à Leonor

Em memória da minha Avó,
Maria dos Anjos Valente (1927-2017)

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Resumo

O gorgulho-do-eucalipto, *Gonipterus platensis* (Marelli) (Coleoptera: Curculionidae), originário da Austrália, é uma das principais pragas de eucaliptos. Sendo uma espécie não nativa, o controlo biológico clássico, pela introdução de inimigos naturais provenientes da sua região de origem, constitui uma estratégia de controlo viável. Esta tem sido a principal medida para controlar esta praga a nível mundial, através da introdução do parasitóide oófago *Anaphes nitens* (Girault) (Hymenoptera: Mymaridae). Porém, este inimigo natural não é totalmente eficaz a reduzir as populações de *G. platensis*, não evitando a ocorrência de prejuízos em diversas regiões, pelo que é relevante identificar alternativas eficazes de controlo para estas áreas. Neste trabalho, foi estimado o impacto económico da praga e o benefício do controlo biológico com *A. nitens* nos últimos 20 anos, usando Portugal como caso de estudo. Os resultados da análise económica mostraram que, na ausência de controlo biológico, as perdas causadas por *G. platensis* seriam quatro vezes ou mais superiores às que ocorrem com *A. nitens*, ainda que esse controlo seja incompleto. Dada a insuficiente eficácia de *A. nitens*, foi avaliada a existência de outros inimigos naturais australianos, que pudessem ser integrados num programa de controlo biológico clássico. De um conjunto de oito inimigos naturais identificados na Tasmânia, o parasitóide oófago *Anaphes inexpectatus* Huber and Prinsloo (Hymenoptera: Mymaridae) foi selecionado para mais estudos. Foi feito um estudo laboratorial comparando a biologia de *A. inexpectatus* e *A. nitens* a diferentes temperaturas, um estudo de competição entre estas duas espécies e uma análise de risco à introdução de *A. inexpectatus* na Península Ibérica. Os resultados sugerem que *A. inexpectatus* é um agente de controlo biológico promissor, que poderá complementar o parasitismo por *A. nitens* em condições de campo, sem afetar a fauna nativa.

Palavras-chave

Anaphes nitens, *Anaphes inexpectatus*, impacto económico, *Eucalyptus globulus*, praga florestal.

Abstract

The Australian weevil *Gonipterus platensis* (Marelli) (Coleoptera: Curculionidae), commonly known as the *Eucalyptus* snout-beetle, is one of the main pests of eucalypts. Because this is a non-native species, classical biological control with natural enemies from its region of origin should be a viable control strategy. The introduction of the Australian parasitoid *Anaphes nitens* (Girault) (Hymenoptera: Mymaridae) has been the main method to control the pest worldwide. However, this natural enemy is not completely effective in reducing *G. platensis* populations and does not avoid the occurrence of damage in several regions. Therefore, it is important to identify effective control alternatives for these areas. In this work, the economic impact of the pest and the benefit of biological control with *A. nitens* over the last 20 years were assessed, using Portugal as a case study. The results of the economic analysis showed that, without biological control, the losses caused by *G. platensis* would be at least four times higher than those occurring with partial control by *A. nitens*. Given the insufficient efficacy of *A. nitens*, the existence of other natural enemies in Australia that could be used in a classical biological control programme was evaluated. From a set of eight natural enemies identified in Tasmania, the egg parasitoid *Anaphes inexpectatus* Huber and Prinsloo (Hymenoptera: Mymaridae) was selected for further studies. Laboratory studies comparing the biology of *A. inexpectatus* and *A. nitens* at different temperatures, a competition study between these two species, and a risk analysis for the introduction of *A. inexpectatus* in the Iberian Peninsula were carried out. Overall results suggest that *A. inexpectatus* might complement parasitism by *A. nitens* under field conditions without non-target effects on native fauna.

Keywords

Anaphes nitens, *Anaphes inexpectatus*, economic impact, *Eucalyptus globulus*, forest pest.

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General Introduction



Importance of eucalypts

Native to Australia and some Pacific islands, eucalypts (genera *Eucalyptus*, *Corymbia*, and *Angophora*, from family Myrtaceae) include more than 800 species, over 740 belonging to the genus *Eucalyptus*. Eucalypts started to be cultivated outside their native range in the 18th century, as botanical curiosities and ornamentals in botanical gardens and arboreta in Europe (Rejmánek and Richardson 2011). Several eucalypt species become widely planted due to their fast growth, good adaptation to a wide range of environmental conditions, and for their wood and non-wood products (Rejmánek and Richardson 2011; Hurley et al. 2016). Nowadays, eucalypts are among the most widely planted forest trees in the world, second only to pines (*Pinus* spp.) (Rejmánek and Richardson 2011). The area planted with eucalypts has been increasing worldwide and in three decades (from 1980 to 2009) it has expanded more than threefold, to over 20 million hectares (Eldridge et al. 1993; GIT Forestry Consulting 2017).

Eucalypts are mostly planted as sources of pulpwood, timber, and firewood, but eucalypt plantations can provide other provisioning services, such as essential oils or foliage for the cut-flower industry (Rejmánek and Richardson 2011; Branco et al. 2015). Additionally, eucalypts provide regulating and supporting services, such as erosion and flood mitigation, and several cultural services due to their recreational and aesthetic value (Branco et al. 2015). In California (USA), for example, the economic value of eucalypts in urban areas as ornamental trees, the most important use for eucalypts in the region, was estimated as ca. 6 000 US dollars per tree (Paine et al. 2015).

Eucalyptus globulus Labill., *E. camaldulensis* Dehnh., *E. grandis* W.Hill, and *E. tereticornis* Sm. are among the most commonly cultivated eucalypt species in the world (Rejmánek and Richardson 2011). *Eucalyptus globulus* is the major pulpwood species planted in temperate regions and the most cultivated in Europe, where it covers 1.3 million ha of forested area (Cerasoli et al. 2016). In Europe, Portugal is the country with the largest area planted with *E. globulus*, covering 812 thousand ha (ICNF 2013). *Eucalyptus globulus* stands provide wood that is used as raw material by the Portuguese pulp and paper companies. This industry has a high socio-economic importance, as it represents 5% of the country's exports, valued at ca. 2 500M euros in 2015, contributes with 4.4% to the Portuguese GDP (gross domestic

product), and also assures about three thousand direct jobs and several thousand indirect jobs (CELPA 2016; INE 2016; EUROSTAT 2017).

Eucalypt pests

Eucalypts early established outside their native range have typically benefited from a pest-free environment, since they were free from their natural phytophagous insects (Wingfield et al. 2008; Hurley et al. 2016). However, over time several non-native insects specific to eucalypts have arrived in areas where these trees are commercially planted (Wingfield et al. 2008; Paine et al. 2011; Hurley et al. 2016). The pathways leading to introductions of insect pests feeding on eucalypts are unknown in most cases, but transport of people and trade of wood products, live plant material, cut branches and other commodities may have contributed to insect's spread (Hurley et al. 2016). Presently, there are 42 eucalypt insect pests from Australia recorded outside their native range (Hurley et al. 2016).

In addition to the pests sharing their native range with that of eucalypts, some pests have originated from plants occurring naturally in the areas into which eucalypts have been introduced (Wingfield et al. 2008). Shifting of native insects onto eucalypts has occurred mainly in Africa, Asia, and South America (Paine et al. 2011). These pests are generally either highly polyphagous or have native Myrtaceae as natural hosts (Paine et al. 2011). For example, the Brazilian moth *Thyriniteina arnobia* Stoll (Lepidoptera: Geometridae) is an important eucalypt pest having several Myrtaceae as native hosts (Lemos et al. 1999). The South American ants *Atta* spp. and *Acromyrmex* spp. (Hymenoptera: Formicidae) and the South African moth *Coryphodema tristis* (Drury) (Lepidoptera: Cossidae) are examples of highly polyphagous species attacking eucalypts (Boreham 2006; Zanetti et al. 2014).

The *Eucalyptus* snout beetle, *Gonipterus platensis*

Weevils belonging to the genus *Gonipterus* (Coleoptera: Curculionidae) are among the most important eucalypt pests (Tooke 1955; Loch 2008; Mapondera et al. 2012; Reis et al. 2012). *Gonipterus* spp. adults and larvae feed on newly expanded leaves, shoots, and buds, causing defoliation and reduction in wood productivity (Reis et al.

2012). There are approximately 20 described species within this genus, all of them native to Australia. Three species, *G. platensis*, *G. pulverulentus*, and an undescribed species have established outside their native range (Mapondera et al. 2012). Until the publication of Mapondera et al. (2012), clarifying the taxonomy of the genus *Gonipterus*, these species have been confused in literature. Both *G. platensis* and the undescribed weevil were referred to as *G. scutellatus*, and *G. pulverulentus* was referred to as *G. gibberus* (EPPO 2005; Mapondera et al. 2012).

Among the three invasive species, all commonly known as *Eucalyptus* snout beetles, *G. platensis* has the widest distribution outside its native range. Native to Tasmania, this species was accidentally introduced into Western Australia, New Zealand, Europe (Portugal and Spain), South America (Argentina, Brazil, and Chile), and USA (California and Hawaii) (Mapondera et al. 2012). Defoliation by *G. platensis* causes eucalypts to lose apical dominance and severely affects yield. In *E. globulus* plantations in Portugal, for example, the snout beetle is reported to cause up to 86% in wood loss (Reis et al. 2012).

Strategies to manage eucalypt pests

The application of insecticides, the selection and planting of resistant eucalypt genotypes, and classical biological control (CBC), i.e. the introduction of non-native natural enemies of a pest aiming at its permanent control, have been the main strategies used to manage invasive eucalypt pests (Hurley et al. 2016).

Chemical control is an effective strategy against some eucalypt pests and it has been used to control *G. platensis* in Portugal (ICNF 2015), Spain (MAPAMA 2017), Chile (Lanfranco and Dungey 2001), and Southwestern Australia (Loch 2005; Loch and Matsuki 2010). Other examples of eucalypt pests controlled with insecticides are *Mnesampela privata* Guenée (Lepidoptera: Geometridae) in Australia (Rapley et al. 2009), *Thaumastocoris peregrinus* Carpintero and Dellapé (Hemiptera: Thaumastocoridae) in Australia and New Zealand (Noack et al. 2009; Murray and Lin 2017), leaf-cutting ants belonging to the genera *Atta* and *Acromyrmex* in Brazil (Zanetti et al. 2014; Lemes et al. 2017), and *Uraba lugens* Walker (Lepidoptera: Nolidae) in New Zealand (Murray and Lin 2017). However, the use of insecticides

against eucalypt pests has been limited, due to the high application costs and to the increasing pressure from certification bodies, such as FSC (Forest Stewardship Council, www.fsc.org), to reduce the area treated with pesticides and the number of products in certified forests (Hurley et al. 2016; FSC 2017; Lemes et al. 2017). Insecticides also present some risks by comparison to biological control and to the deployment of resistant eucalypts, such as risks to human health, domestic animals, and to the environment, including effects on beneficial natural enemies and pollinators (Pimentel et al. 1992; Sexton et al. 2007).

Variability among eucalypts on the susceptibility to phytophagous insects has been studied for several pests and in some cases employed in breeding or forest management programs. The selection and breeding of resistant eucalypts (species, hybrids, provenances, families, and clones) has been a valuable tool to deal with pest problems (Wingfield et al. 2013). Examples of target pests for which eucalypt susceptibility has been investigated are *Glycaspis brimblecombei* Moore (Hemiptera: Psyllidae) (Brennan et al. 2001), *M. privata* (Jones et al. 2002), *Atta laevigata* (Smith) and *Atta sexdens* Linnaeus (Santana et al. 1989), *Phoracantha semipunctata* (Fabricius) (Coleoptera: Cerambycidae) (Hanks et al. 1995), and *Leptocybe invasa* Fisher and La Salle (Hymenoptera: Eulophidae) (Kulkarni 2010).

In countries where *G. platensis* is present, *E. globulus* is consistently considered a preferred host (Hanks et al. 2000; Lanfranco and Dungey 2001; Reis et al. 2012), but resistant eucalypts have also been identified. *Eucalyptus fastigata* H.Deane and Maiden, *E. obliqua* L'Her., and *E. amygdalina* Labill. are some examples of *Eucalyptus* species that are completely avoided by *G. platensis* in the field (Cordero-Rivera and Santolamazza-Carbone 2000). *Eucalyptus nitens* Maiden, a species that is also less attacked by the snout beetle, has been widely planted as alternative to *E. globulus* in cooler northern regions of Spain where severe defoliation by *G. platensis* repeatedly occurs (Pérez-Cruzado et al. 2011). However, *E. nitens* has important disadvantages when compared to *E. globulus*, such as poor coppicing ability (Little et al. 2002) and lower wood quality for the pulp and paper industry (Kibblewhite et al. 2001).

CBC has been an important strategy to deal with eucalypt pest problems. CBC applied to the *Eucalyptus* snout beetle, *G. platensis*, is the main subject of the

present thesis and, due to its relevance, three sections dedicated to CBC are presented below: a section on CBC in general, a section on CBC of eucalypt pests, and a section on CBC of *G. platensis*.

Classical biological control

CBC is a particularly useful strategy to manage invasive species that are not controlled by natural enemies in the invaded range (Wingfield et al. 2015; Kenis et al. 2017). Until the end of 2010, 2 384 species of natural enemies had been introduced worldwide for CBC of insect pests, leading to the control of 172 of 588 target pests (Cock et al. 2016). Invasive forest pests, in particular, have been a priority target for CBC, in part because other management methods that are practiced in agricultural systems are not so appropriate in forests. The use of insecticides, for example, is increasingly banned in many forested areas. The development of resistant plant varieties, although effective and used in integrated pest management in eucalypts, is a slow process and usually not a short or medium term option (Kenis et al. 2017).

Complete control of the target pest is achieved in some cases, but frequently success is only partial or the introduced CBC agent does not establish after release (Cock et al. 2016; Hajek et al. 2016; Kenis et al. 2017). However, the rate of successes has increased in the last decades, in part due to a more judicious selection of CBC agents, based on pre-introduction studies (Cock et al. 2016; Hajek et al. 2016).

Implementing CBC is not a simple task and may require significant work, time, and financial resources, with costs generally including the labour and materials associated with surveying, importation, quarantine, release and distribution of the natural enemies, verification of establishment, and evaluation of efficacy (Naranjo et al. 2015). Moreover, importing and releasing exotic natural enemies entail risks of undesirable non-target impacts, including changes in the distribution and abundance of native species (van Lenteren et al. 2006; De Clercq et al. 2011; Simberloff 2012). Even though the majority of insects used worldwide in CBC have been shown to be safe (van Lenteren et al. 2006; Hajek et al. 2016), there are examples of introduced natural enemies of pests that have caused negative effects on non-target organisms (Howarth 1991; Louda et al. 2003). To reduce the risk of non-target effects, a

thorough risk assessment should be performed before introducing a CBC agent into a new region (van Lenteren et al. 2006; Barratt 2011). A successful and safe programme should include the following steps (modified from Kenis et al. 2017):

- Gather the available information on the pest (identification of the pest and its region of origin; assessment of its economic and ecological impact);
- Gather the available information on the natural enemies of the pest (literature surveys on the natural enemies and on previous CBC projects; field surveys for natural enemies in the invaded range, to identify species already present and empty ecological niches);
- Select the region where to search for candidate CBC agents (based on the pest's native range, climate similarities with the area of introduction, and practicality of surveying);
- Identify stakeholders and establish collaborations between the region of origin and the region of introduction;
- Apply for permission to import the natural enemies;
- Collect the natural enemies in the native range and, when possible, gather information on their role as mortality factors of the pest and on their biology and ecology, particularly on host range;
- Import the natural enemies to the country of introduction and establish colonies in a certified quarantine facility;
- Study the efficacy, host specificity, and biological parameters of the natural enemies in quarantine conditions;
- Examine the available information and select the most suitable natural enemy or enemies for release;
- Apply for permission to release the selected natural enemies as CBC agents into the field;
- Develop rearing procedures and release methods, including the identification of areas suitable for release and monitoring;
- Release the CBC agents in selected sites, with the aim to cover the pest's area of distribution;
- Monitor the establishment of the CBC agents, evaluate their impact on the pest population and test for non-target effects.

Several of these steps, applied to CBC of *G. platensis*, are addressed in the present work.

Classical biological control of eucalypt pests

CBC has been applied to eucalypts since 1905, when *Rhyzobius ventralis* (Erichson) (Coleoptera: Coccinellidae) was introduced into New Zealand to control the gum-tree scale, *Eriococcus coriaceus* Maskell (Hemiptera: Eriococcidae) (Cameron et al. 1993; Withers 2001). From our review, at least 37 Australian natural enemies were used as CBC agents against eucalypt pests (Table 1.1)¹. More than 90% of these natural enemies are parasitoids and 86% belong to the order Hymenoptera. About half of them have provided moderate to high control of the target pest, while the degree of success of almost 20% is unknown. Examples of complete successful CBC programmes of eucalypt pests include the control of *Ctenarytaina eucalypti* (Maskell) (Hemiptera: Psyllidae) with the parasitoid *Psyllaephagus pilosus* Noyes in California, Britain, France, and Eire (Hodkinson 1999), and the control of *P. semipunctata* with *Avetianella longoi* Siscaro (Hymenoptera: Encyrtidae) in California (Paine et al. 2015).

¹ Natural enemies that were not confirmed to be native from Australia were not considered in this review. For example, *Psyllaephagus blastopsyllae* Tamesse et al. (Hymenoptera: Encyrtidae), a parasitoid that was found attacking *Blastopsylla occidentalis* Taylor (Psyllidae, Spondyliaspidae) in Cameroon and South Africa, was not included in Table 1.1 as it is probably of African origin (Tamesse et al. 2014; Bush et al. 2016).

Table 1.1 Australian natural enemies of eucalypt pests introduced in other regions as classical biological control agents. Regions: Africa (Af); Asia (As); Europe (E), North America (NA), Oceania (O; other than Australia and particularly referring to New Zealand) and South America (SA). Type of introduction: accidental (A) or intentional (I).

Natural enemy	Order: family of natural enemy	Date, region, and type of introduction	Natural enemy guild	Target pest	Order: family of target pest	Success of control	References
<i>Anaphes inexpectatus</i>	Hym: Mymaridae	2012, E (I)	Egg parasitoid	<i>Gonipterus platensis</i>	Col: Curculionidae	Unknown	Valente et al. 2012
<i>Anaphes nitens</i>	Hym: Mymaridae	1926, Af (I) 1927, NZ (I) 1927, SA (A) 1978, E (I) 1994, NA (I)	Egg parasitoid	<i>Gonipterus platensis</i> (E, NA, NZ, SA) <i>Gonipterus pulverulentus</i> (SA) <i>Gonipterus</i> sp. 2 ^a (E, Af)	Col: Curculionidae	Moderate to High	Tooke 1955; Pinet 1986; Sanches 2000; Withers 2001; Paine and Millar 2002; Otero et al. 2003
<i>Anaphes tasmaniae</i>	Hym: Mymaridae	2009, SA (I)	Egg parasitoid	<i>Gonipterus platensis</i>	Col: Curculionidae	Unknown	Mayorga et al. 2013
<i>Avetianella longoi</i>	Hym: Encyrtidae	1991, E (A) 1993, Af, NA (I) 2000, SA (I)	Egg parasitoid	<i>Phoracantha semipunctata</i> <i>Phoracantha recurva</i>	Col: Cerambycidae	High on <i>P. semipunctata</i> ; Moderate on <i>P. recurva</i>	Siscaro 1992; Lanfranco and Dungey 2001; Paine and Millar 2002; Paine et al. 2015
<i>Bracon phylacteophagus</i>	Hym: Braconidae	1988, NZ (I)	Larval parasitoid	<i>Phylacteophaga froggatti</i>	Hym: Pergidae	High	Faulds and others 1991; Withers 2001
<i>Cleobora mellyi</i>	Col: Coccinelidae	1979, NZ (I)	Predator	<i>Paropsis charybdis</i>	Col: Chrysomelidae	Low	Bain and Kay 1989; Murray et al. 2008
<i>Cleruchoides noackae</i>	Hym: Mymaridae	2009, SA (I)	Egg parasitoid	<i>Thaumastocoris peregrinus</i>	Hem: Thaumastocoridae	High	Mutitu et al. 2013; Barbosa et al. 2017
<i>Closterocerus chamaeleon</i>	Hym: Eulophidae	2005, As (I) 2006, E (I) 2012, SA (A) 2015, Af (A)	Larval and pupal parasitoid	<i>Ophelimus maskelli</i>	Hym: Eulophidae	High	Protasov et al. 2007; Rizzo et al. 2015; Bush et al. 2016; Mendel et al. 2017
<i>Cotesia urabae</i>	Hym: Braconidae	2011, NZ (I)	Larval parasitoid	<i>Uraba lugens</i>	Lep: Nolidae	Unknown	Avila et al. 2013
<i>Enoggera nassau</i>	Hym: Pteromalidae	1986, Af (I) 1987, NZ (I)	Egg parasitoid	<i>Trachymela tincticollis</i> (Af) <i>Paropsis charybdis</i> (NZ)	Col: Chrysomelidae	Failed to establish in Af Moderate in NZ	Tribe 2000; Murray et al. 2008
<i>Enoggera reticulate</i>	Hym: Pteromalidae	1986, Af (I) 2000, NA (I)	Egg parasitoid	<i>Trachymela tincticollis</i> (Af)	Col: Chrysomelidae		Tribe 2000; Paine and Millar 2002; Paine et al.

Chapter 1: General Introduction

Natural enemy	Order: family of natural enemy	Date, region, and type of introduction	Natural enemy guild	Target pest	Order: family of target pest	Success of control	References
				<i>Trachymela sloanei</i> (NA)			2015
<i>Froggattimyia tillyardi</i>	Dip: Tachinidae	1975, NZ (I)	Larval parasitoid	<i>Paropsis charybdis</i>	Col: Chrysomelidae	Failed to establish	Murray et al. 2008
<i>Jarra maculipennis</i>	Hym: Braconidae	1995, Af (I)	Larval parasitoid	<i>Phoracantha semipunctata</i> <i>Phoracantha recurva</i>	Col: Cerambycidae	Failed to establish	Tribe 2003
<i>Jarra phoracantha</i>	Hym: Braconidae	1995, Af (I) 1997, NA (I)	Larval parasitoid	<i>Phoracantha semipunctata</i> <i>Phoracantha recurva</i>	Col: Cerambycidae	Unknown	Paine and Millar 2002; Tribe 2003
<i>Megalyra fasciipennis</i>	Hym: Megalyridae	1910, Af (I)	Pupal parasitoid	<i>Phoracantha semipunctata</i> <i>Phoracantha recurva</i>	Col: Cerambycidae	Moderate ^b	Moore 1993; Tribe 2003
<i>Megastigmus lawsoni</i>	Hym: Torymidae	2007, As (I)	Larval parasitoid	<i>Leptocybe invasa</i>	Hym: Eulophidae	Low	Doğanlar and Hassan 2010; Mendel et al. 2017
<i>Megastigmus zvimendeli</i>	Hym: Torymidae	2007, As (I)	Larval parasitoid	<i>Leptocybe invasa</i>	Hym: Eulophidae	High	Doğanlar and Hassan 2010; Mendel et al. 2017
<i>Neopolycystus insectifurax</i>	Hym: Pteromalidae	1986, Af (I) 2002, NZ (A)	Egg parasitoid	<i>Trachymela tincticollis</i> (Af) <i>Paropsis charybdis</i> (NZ)	Col: Chrysomelidae	Failed to establish in Af Moderate in NZ	Tribe 2000; Murray et al. 2008
<i>Neopolycystus</i> sp.	Hym: Pteromalidae	1987, NZ (I)	Egg parasitoid	<i>Paropsis charybdis</i>	Col: Chrysomelidae	Failed to establish	Murray et al. 2008
<i>Orchus chalybeus</i>	Col: Coccinelidae	1905, NZ (I) ^c	Predator	<i>Eriococcus coriaceus</i>	Hem: Eriococcidae	Low	Morales and Bain 1989
<i>Procheiloneurus</i> sp.	Hym: Encyrtidae	1986, Af (I)	Egg parasitoid	<i>Trachymela tincticollis</i>	Col: Chrysomelidae	Failed to establish	Tribe 2000
<i>Pseudoleucopsis benefica</i>	Dip: Chamaemyiidae	1932, NZ (I)	Predator	<i>Eriococcus coriaceus</i>	Hem: Eriococcidae	Failed to establish ^d	Morales and Bain 1989
<i>Psyllaephagus bliteus</i>	Hym: Encyrtidae	2000, NZ (A) 2000, NA (I) 2003, SA (A) 2011, E (A) 2015, Af (A)	Nymphal parasitoid	<i>Glycaspis granulate</i> (NZ) <i>Glycaspis brimblecombei</i>	Hem: Aphalaridae	Moderate to High	Withers 2001; Dahlsten et al. 2005; Caleca et al. 2011; Ferreira Filho et al. 2015; Bush et al. 2016
<i>Psyllaephagus gemitus</i>	Hym: Encyrtidae	1999, NZ (A)	Nymphal parasitoid	<i>Cardiaspina fiscella</i>	Hem: Aphalaridae	High	Withers 2001
<i>Psyllaephagus parvus</i>	Hym: Encyrtidae	2007, NA (A)	Nymphal parasitoid	<i>Eucalyptolyma maideni</i>	Hem: Aphalaridae	Moderate	Jones et al. 2011; Paine et al. 2015
<i>Psyllaephagus perplexans</i>	Hym: Encyrtidae	2007, NA (A)	Nymphal parasitoid	<i>Cryptoneossa triangula</i>	Hem: Aphalaridae	Moderate	Jones et al. 2011; Paine et al. 2015
<i>Psyllaephagus</i>	Hym: Encyrtidae	1889, NZ (A)	Nymphal	<i>Ctenarytaina eucalypti</i>	Hem:	High	Dahlsten et al. 1998;

Chapter 1: General Introduction

Natural enemy	Order: family of natural enemy	Date, region, and type of introduction	Natural enemy guild	Target pest	Order: family of target pest	Success of control	References
<i>pilosus</i>		1993, NA (I) 1994, E (I) 2000, SA (A)	parasitoid		Aphalaridae		Withers 2001; Chauzat et al. 2002; Santana and Burckhardt 2007
<i>Psyllaephagus richardhenryi</i>	Hym: Encyrtidae	2002, NZ (A)	Nymphal parasitoid	Several psyllids	Hem: Aphalaridae	Unknown	Berry 2007
<i>Quadrastichus mendeli</i>	Hym: Eulophidae	2007, As (I) 2016, E, Af (A)	Larval parasitoid	<i>Leptocybe invasa</i>	Hym: Eulophidae	High	Kim et al. 2008; Nugnes et al. 2016; Zheng et al. 2016; Bush et al. 2017; Mendel et al. 2017
<i>Rhyzobius ventralis</i>	Col: Coccinellidae	1905, NZ (I) ^c	Predator	<i>Eriococcus coriaceus</i>	Hem: Eriococcidae	High	Cameron et al. 1993; Withers 2001
<i>Selitrichodes kryceri</i>	Hym: Eulophidae	2007, As (I)	Larval parasitoid	<i>Leptocybe invasa</i>	Hym: Eulophidae	Low	Kim et al. 2008; Mendel et al. 2017
<i>Selitrichodes neseri</i>	Hym: Eulophidae	2012, Af (I) 2015, SA (I)	Larval and pupal parasitoid ^e	<i>Leptocybe invasa</i>	Hym: Eulophidae	Unknown (established in Af and SA)	Zheng et al. 2014; Masson et al. 2017
<i>Stathmopoda melanochra</i>	Lep: Oecophoridae	1932, NZ (I)	Predator	<i>Eriococcus coriaceus</i>	Hem: Eriococcidae	Unknown	Cameron et al. 1993
<i>Stethynium breviovipositor</i>	Hym: Mymaridae	2005, As (I)	Larval parasitoid	<i>Ophelimus maskelli</i>	Hym: Eulophidae	Low	Huber et al. 2006; Mendel et al. 2017
<i>Stethynium ophelimi</i>	Hym: Mymaridae	2005, As (I)	Larval parasitoid	<i>Ophelimus maskelli</i>	Hym: Eulophidae	Moderate	Huber et al. 2006; Mendel et al. 2017
<i>Syngaster lepidus</i>	Hym: Braconidae	1969, 1995 ^f , Af (I) 1997, NA (I)	Larval parasitoid	<i>Phoracantha semipunctata</i> <i>Phoracantha recurva</i> <i>Strepsicrates</i> (= <i>Stictea</i>) <i>macropetana</i>	Col: Cerambycidae	Failed to establish in 1969 Moderate	Hanks et al. 1996; Tribe 2003
<i>Trigonospila brevifacies</i>	Dip: Tachinidae	1967, NZ (I)	Larval parasitoid		Lep: Tortricidae	High	Green 1984; Withers 2001

^a *Sensu* Mapondera et al. (2012).

^b *Megalyra fasciipennis* was introduced into South Africa in 1910, but it remained undetected until 1962. A survey performed in 1993 recorded a parasitism rate of 52.5% by *M. fasciipennis* (Tribe 2003).

^c According to Morales and Bain (1989), *Orcus chalybeus* and *Rhyzobius ventralis* were introduced into New Zealand in 1899 to control *Saissetia oleae* (Hemiptera: Coccidae), prior to *Eriococcus coriaceus* being recognised as a problem.

^d Only 11 adults of *Pseudoleucopsis benefica* were released into New Zealand (Morales and Bain 1989).

^e *Selitrichodes neseri* is also able to parasitise callow adults (Dittrich-Schröder et al. 2014).

^f *Syngaster lepidus* was introduced into South Africa in 1969, but failed to establish. A second attempt was performed in 1995 and the parasitoid became established in the Tzaneen district (Tribe 2003).

Classical biological control of *Gonipterus platensis*

CBC with the egg parasitoid *Anaphes nitens* (Hymenoptera: Mymaridae) has been the main strategy to manage *G. platensis* and other *Gonipterus* species worldwide. This natural enemy, native to Australian mainland, was first used in 1926, in South Africa (Tooke 1955). It was also introduced into other African countries, New Zealand, South America, the USA, and Europe (Marelli 1939; Frappa 1950; Williams et al. 1951; Arzone and Vidano 1978; Huber and Prinsloo 1990; Hanks et al. 2000; Lanfranco and Dungey 2001). In several regions, *A. nitens* has brought *Gonipterus* spp. populations under control, reducing damage to insignificant levels within a few years (Kevan 1946; Tooke 1955; Hanks et al. 2000; Valente et al. 2004). In South Africa, the parasitoid was so successful that a memorial to this biological control programme was erected in the Province of KwaZulu-Natal (Londt 1996).

Despite the good results obtained with *A. nitens* in many regions, successful control has not been achieved everywhere, especially in some regions of South America (Gumovsky et al. 2015), Western Australia (Loch 2008), and Southwestern Europe (Cordero-Rivera et al. 1999; Valente et al. 2004; Reis et al. 2012). Different climatic requirements of *A. nitens* and *G. platensis* and asynchrony between oviposition by the snout beetle and the parasitoid may explain the insufficient efficacy of biological control (Tribe 2003; Loch 2008; Reis et al. 2012). In cold regions, foliage flushing by eucalypt trees is inhibited by low temperatures during the winter months, which reduces the availability of adequate oviposition sites for *G. platensis* females and consequently decreases the number of hosts available for *A. nitens* (Tooke 1955; Tribe 2003; Loch 2008). This, in turn, causes *A. nitens* populations to decrease during the winter. In late winter/early spring, when oviposition by *G. platensis* starts to increase, *A. nitens* is unable to respond in adequate numbers and to provide high mortality rates. Even though parasitism rates in late spring may surpass 90%, the snout beetle larvae escaping parasitism early in the season have already caused defoliation (Tooke 1955; Cordero-Rivera et al. 1999; Tribe 2003; Loch 2008; Reis et al. 2012). In Portugal, Reis et al. (2012) found a positive correlation between both parasitism rates by *A. nitens* in late winter/early spring and maximum temperature of the winter months (MaxTw), by studying 34 *E. globulus* plantations ranging from 290 m to 900 m in elevation. In regions with MaxTw below 10 °C, parasitism ranged

from 0% to 45%, while for MaxTw equal or above 12 °C parasitism ranged between 50% and 100%, suggesting that *A. nitens* is ineffective at low temperatures.

Because *A. nitens* has not provided successful control in several important regions of eucalypt production, CBC with other natural enemies should be considered. Several natural enemies of *Gonipterus* spp. have been reported from Australia. Apart from *A. nitens*, two wasps, *Euderus* sp. (Hymenoptera: Eulophidae) and *Centrodora* sp. (Hymenoptera: Aphelinidae) were reared from the eggs of *G. platensis* in Southwestern Australia (Loch 2008). In the same study, an unidentified tachinid fly (Diptera: Tachinidae) was reared from the larvae of *G. platensis*. Tooke (1955) also reported an unidentified tachinid in South Australia and in New South Wales. In Tasmania, Tribe (2003) reported the occurrence of the larval parasitoids *Oxyserphus turneri* (Dodd) (Hymenoptera: Proctotrupidae), *Apanteles* sp. (Hymenoptera: Braconidae), and an unidentified tachinid. In 2011, the larval parasitoid *Entedon magnificus* (Girault and Dodd) (Hymenoptera: Eulophidae) was collected in Tasmania from *Gonipterus* spp. and shown to successfully parasitise *G. platensis* (Gumovsky et al. 2015). The egg parasitoids *Anaphes tasmaniae* Huber and Prinsloo, *Anaphes inexpectatus* Huber and Prinsloo, and *Centrodora damoni* (Girault) (Hymenoptera: Aphelinidae) are known to occur in Tasmania (Huber and Prinsloo 1990; Tribe 2003; Ward et al. 2016). *Centrodora damoni* was also reported from Queensland and Canberra (Ward et al. 2016).

Objectives

In the present thesis, research deals with several steps of CBC applied to the invasive pest *G. platensis*. The main objectives of this study, which are addressed in different chapters, are to:

- assess the economic importance of *G. platensis* and the economic benefits resulting from a CBC programme targeting this pest (Chapter 2);
- identify the main parasitoids attacking *G. platensis* in its native range and compare life history traits of *A. inexpectatus* and *A. nitens* at different temperatures (Chapter 3);

- evaluate the competitive interactions among *A. nitens* and *A. inexpectatus* and assess their outcome (Chapter 4);
- evaluate the risk of non-target effects resulting from releasing *A. inexpectatus* in the Iberian Peninsula (Chapter 5).

In **Chapter 2, “Economic outcome of classical biological control: a case study on the *Eucalyptus* snout beetle *Gonipterus platensis* and the parasitoid *Anaphes nitens*”**, an assessment is made of: i) the economic impact of *G. platensis* in *E. globulus* plantations, using Portugal as a case study; ii) the economic benefits from partial control of *G. platensis* by *A. nitens*, by comparing current losses with the expected losses of eucalypt wood under three hypothetical scenarios without biological control, during a 20-year period; and iii) the economic outcome of the biological control programme conducted in Portugal aiming to anticipate the effects of *A. nitens*.

In **Chapter 3, “Pre-selection and biological potential of the egg parasitoid *Anaphes inexpectatus* for the control of the *Eucalyptus* snout beetle, *Gonipterus platensis*”**, field surveys conducted in the pest’s native range (Tasmania, in Australia) aiming to search for natural enemies of *G. platensis* are described. Based on data collected in the native range and in the laboratory, the egg parasitoid *A. inexpectatus* was selected for pre-release studies. Life history traits of *A. inexpectatus* and *A. nitens* are compared at six temperatures (5, 10, 15, 20, 25, and 30 °C), including development times, thermal constants, viability, parasitism, and behaviour.

In **Chapter 4, “Assessing the competitive interactions between two egg parasitoids of the *Eucalyptus* snout beetle, *Gonipterus platensis*, and their implications for biological control”**, laboratory studies to assess intra- and interspecific competition in *A. nitens* and *A. inexpectatus* are presented. Also, the effect of *G. platensis* egg age on host acceptance and suitability for parasitoid development is performed for both *Anaphes* species. The results are discussed and predictions are made on the outcome of competitive interactions between these two parasitoids under field conditions.

In Chapter 5, “Environmental risk assessment of the egg parasitoid *Anaphes inexpectatus* for classical biological control of the *Eucalyptus* snout beetle, *Gonipterus platensis*”, potential non-target effects of *A. inexpectatus* are assessed. No-choice tests are conducted with 17 non-target species to assess host specificity, including 11 curculionids. Based on the host specificity test results and the potential host fauna found in the target area (Iberian Peninsula), the environmental risk of introducing *A. inexpectatus* is discussed.

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CHAPTER 2

Economic outcome of classical biological control: a case study on the *Eucalyptus* snout beetle *Gonipterus platensis* and the parasitoid *Anaphes nitens*

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Economic outcome of classical biological control: a case study on the *Eucalyptus* snout beetle *Gonipterus platensis* and the parasitoid *Anaphes nitens*

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Abstract

Despite the importance of invasive pests, few studies address the cost-benefit of the strategies used to control them. The present work aims to assess the economic impact of the *Eucalyptus* snout beetle, *Gonipterus platensis* (Coleoptera: Curculionidae) and the benefits resulting from its biological control with the egg parasitoid *Anaphes nitens* (Hymenoptera: Mymaridae) in Portugal, over a 20-year period. Comparisons were made between the real situation (with parasitism) and three scenarios without biological control: 1) replacement of the susceptible *Eucalyptus globulus* by resistant species; 2) insecticide use; and 3) offset of yield losses by imported wood. A cost-benefit analysis was performed to evaluate a programme that aimed to accelerate *A. nitens* establishment. Although *A. nitens* provides adequate pest control in several regions, 46% of the area planted with eucalypts is affected by the beetle, causing wood losses of 648M euros over 20 years. Losses in the three hypothetical scenarios were estimated at 2 451M-7 164M euros, resulting in benefits from biological control of 1 803M-6 516M euros, despite the fact that only partial success was achieved. Anticipating biological control by just one, two, or three years resulted in benefit-cost ratios of 67, 190, and 347, respectively. Because nonmarket values were not accounted for in the calculations, these figures are likely underestimated.

Keywords

Eucalyptus weevil; cost-benefit analysis; biological invasions; pest management; natural enemy; defoliation.

1. Introduction

Invasive alien species pose a major threat to natural and managed ecosystems and can have substantial ecological and economic impacts. Biological invasions by insects alone cost at least 70 billion US dollars per year globally, but this value is greatly underestimated due to the lack of reliable cost assessments (Bradshaw et al. 2016). Classical biological control (CBC) is a particularly useful strategy to manage non-native species that attain pest status in their introduced range due to the absence of natural enemies (Kenis et al. 2017). Between 1870 and 2010, 2 384 species of natural enemies have been introduced for CBC of insect pests worldwide, leading to the control of 172 of 588 target pests (Cock et al. 2016). Despite the high number of programmes undertaken, analyses weighing economic costs and benefits of CBC have hardly been assessed (Greathead 2003; Kenis and Branco 2010; Naranjo et al. 2015). The scarcity of economic studies arises from many causes, including lack of funding for post-release monitoring, long periods from release until full field establishment of the biological control agent, difficulty in assessing impacts of CBC programmes, or difficulty in assigning monetary values to externalities (McFayden 2008; Cock et al. 2015). In addition, when successful control is achieved the problem disappears and the focus shifts to other problems (Paine et al. 2015).

Gonipterus platensis (Marelli) (Coleoptera: Curculionidae) is one of three species from the Australian genus *Gonipterus* that were accidentally introduced in other parts of the world, where they became pests of eucalypts (Mapondera et al. 2012; Hurley et al. 2016). CBC with the egg parasitoid *Anaphes nitens* (Girault) (Hymenoptera: Mymaridae) has been the strategy most commonly used to reduce *Gonipterus* spp. populations. This natural enemy was first used in South Africa, in 1926 (Tooke 1955). It was also introduced in New Zealand, North and South America, and Europe (Tooke 1955; Arzone and Vidano 1978; Hanks et al. 2000). Good results were obtained with *A. nitens* in many countries, but complete success was not always

achieved, especially in the case of *G. platensis* in some regions in South America, Western Australia, and Southwestern Europe (Valente et al. 2004; Loch 2008; Mapondera et al. 2012; Reis et al. 2012).

The present work was conducted in Portugal, which is a relevant country for eucalypt wood production. The Tasmanian blue gum, *Eucalyptus globulus* Labill., is the most extensively planted forest species in the country, covering ca. 812 000 ha (ICNF 2013). This value represents over 50% of the total area occupied by *E. globulus* in Europe and over one fourth of the area planted with this species worldwide (Harwood 2015; Cerasoli et al. 2016). *Eucalyptus globulus* plantations are the main source of raw material for pulp and paper production, one of the most important industries in the country. Despite the high socio-economic importance of eucalypt stands, the vast area occupied by monocultures of this exotic species may be perceived as having negative ecological effects (Veiras and Soto 2011). Similarly to other managed forest plantations, eucalypt stands may be the source of ecosystem disservices and can generate negative externalities, such as competition with other plant species and soil erosion. However, such negative impacts can be effectively avoided by adopting adequate forest design and management practices (Branco et al. 2015). One aspect that has generated much controversy is the invasive potential of eucalypts. Even though a few species have been listed as invasive, eucalypts seldom spread considerable distances from planting sites (see Rejmánek and Richardson, 2011). In recent studies, Fernandes et al. (2016, 2017) showed that *E. globulus* does not display invasive behaviour in Portugal. On the other hand, eucalypt stands can provide many ecosystem services, which have been summarised by Branco et al. (2015).

Prior to the detection of the snout beetle in Portugal, in 1995 (Valente et al. 2004), *A. nitens* had already been introduced in Spain, in 1994 (Pérez Otero et al. 2003). Natural dispersion of *A. nitens* from Spain would probably have been enough to promote the establishment of the parasitoid in Portugal, as there are no relevant geographical barriers between the two neighbouring countries. Nevertheless, a programme to rear and release *A. nitens* in Portugal was launched in 1997, aiming to accelerate the benefits from this biological control agent. Around 300 000 parasitoids were released over a period of four years (1997-2000), after which *A. nitens* rapidly established and, within one year, parasitism rates in some plantations reached up to

80% (Valente et al. 2004). Currently, i.e. 20 years later, *A. nitens* is widely distributed across the country and successful control of *G. platensis* populations has been achieved in several areas. However, in some inland regions of northern and central Portugal, with cooler climate than the southern and coastal areas, the parasitoid remains ineffective (Valente et al. 2004; Reis et al. 2012).

Despite the high economic importance of eucalypts worldwide and the vast distribution of *Gonipterus* spp., little information is currently available on either the economic impact of these insects or the economic benefits resulting from their control. In California, Jetter and Paine (2004) assessed the benefits of controlling *G. platensis* attacking urban trees as the average amount that a household would be willing to pay (*sensu* Boardman et al. 1996) for a public pest control programme. The authors concluded that each household would pay about 21 times more to import and release *A. nitens* than for the implementation of a chemical control programme. Paine et al. (2015) reported complete control of *G. platensis* by *A. nitens* in California, with a benefit-cost ratio ranging from 428 to 1 070 for a total investment of 2.6M US dollars in CBC programmes that targeted the snout beetle and seven other eucalypt pests. In Portugal, Reis et al. (2012) found that defoliation by *G. platensis* severely affects the yield of *E. globulus* plantations, causing up to 86% wood loss in some areas. However, to date, neither the effect of *G. platensis* nor of the parasitoid have been economically assessed.

By assessing the economic impact of this key forest pest and the economics of its biological control, the present case study aims to discuss the importance of weighing costs and benefits of CBC on pest management decision making. The specific objectives of this study were to assess: i) the economic impact of *G. platensis* in *E. globulus* plantations in Portugal; ii) the economic benefits resulting from partial control of *G. platensis* by *A. nitens*, by comparing expected losses of eucalypt wood under three hypothetical scenarios without biological control, over a period of 20 years; and iii) the economic outcome of the biological control programme conducted in Portugal with the aim of anticipating the expected benefits of *A. nitens* natural dispersion.

2. Material and Methods

2.1. Economic impact of *G. platensis* in Portugal

2.1.1. Area affected during the spreading phase

During the dispersion phase of *G. platensis* in Portugal (1996-2003), field surveys were conducted annually to assess the area affected by the snout beetle (as described in Appendix 1).

2.1.2. Damage by *G. platensis*

To assess the area currently affected by the snout beetle, a survey was conducted between 2011 and 2014 over an area of ca. 85 000 ha of *E. globulus* plantations (managed by The Navigator Company) that extended to all Territorial Units of Continental Portugal (see Appendix 2 and Fig. S1). The distribution of *G. platensis* attacks in 2011-2014 was extrapolated per NUTS3 (Nomenclature of Territorial Units for Statistics, version 2010; EUROSTAT 2016) region for the period between 2004 and 2016, using the available national forest inventories (ICNF 2013). According to these inventories, the area planted with eucalypts in Continental Portugal was 717 246 ha in 1995, 785 762 ha in 2005, and 811 943 ha in 2010. Based on these numbers, the total area planted with eucalypts was assumed to be 717 246 ha between 1996 and 2004, 785 762 ha from 2005 to 2009, and 811 943 ha from 2010 to 2016. Because *G. platensis* populations were still establishing between 1996 and 2003 (see Section 2.1.1 and Appendix 1), the economic impact in a given year during this period was assumed to have occurred only in areas already occupied by the insect in the previous year.

2.1.3. Wood loss estimates

The percentage of tradeable wood production loss (*WPL*) was assessed for each defoliation level (see Section 2.1.2) using Eq. (1) (Reis et al., 2012), where *D* is percent defoliation by *G. platensis*:

$$WPL = 5.428e^{0.0027D} \quad \text{Eq. (1)}$$

This equation was developed for conditions similar to those of the present study and is, to the best of our knowledge, the most adequate model available, even though it

probably underestimates wood loss, as stated by its authors. For plantations having Very high defoliation, *WPL* was assumed to be 100% rather than the 72% given by Eq. (1), because even if some biomass is produced it will not have commercial use for pulping (C. Valente, personal observation). Based on this assumption and on the class marks of the defoliation intervals for each level of attack, the following categories of *WPL* were obtained: 100% (Very high defoliation); 42% (High defoliation); 16% (Moderate defoliation); 7% (Low defoliation); and 0% (No damage). Tradeable wood volume lost per year (*WVL*; $\text{m}^3\text{ob}\cdot\text{year}^{-1}$, where ob means over bark) per NUTS3 region was estimated with Eq. (2) by applying *WPL* to the potential annual productivity (*PAP*; $\text{m}^3\text{ob}\cdot\text{year}^{-1}$) for *E. globulus* without defoliation:

$$WVL = WPL \cdot PAP \quad \text{Eq. (2)}$$

PAP was assessed for NUTS3 using 3PG model (Landsberg and Waring, 1997) parametrised with unpublished data from The Navigator Company for *E. globulus*. The model ran with soil data collected in each plantation [stoniness, soil texture, soil depth, and suitability class for *E. globulus* according to Sousa et al. (2013)] and climate data provided by the Portuguese Meteorological Institute (Instituto Português do Mar e da Atmosfera) [average monthly rainfall, average monthly minimum temperature and average monthly maximum temperature, from the climate normal of 1961-2000; average annual radiation and average number of days with rainfall, from the climate normal of 1941-1970]. Model outputs were obtained from 10 669 records, corresponding to ca. 120 000 ha distributed throughout the country. Mean annual tradeable wood increment (*MAI*; $\text{m}^3\text{ob}\cdot\text{ha}^{-1}\cdot\text{year}^{-1}$) estimated by 3PG for each soil-climate combination was used to determine the average potential *MAI* for NUTS3 in a scenario without defoliation. Total *PAP* per NUTS3 region was calculated by multiplying *MAI* in each region by the corresponding number of hectares planted with eucalypts.

2.1.4. Economic loss estimates

To assess the annual economic impact of *G. platensis*, *WVL* estimates for each year were converted into monetary units (euros) using stumpage prices (i.e. wood prices before harvesting and transportation to the mill; euros. m^{-3}ob). Because the domestic price of eucalypt wood is usually lower than the f.o.b. price (“free on board”, i.e. the price of an imported good at the border) and higher than the c.i.f. price (“cost,

insurance and freight”, i.e. the price of an exported good at the border), wood was considered to be a non-tradeable commodity and was therefore valued at domestic prices in the analyses, as recommended by Campbell and Brown (2003). Annual stumpage prices from 1997 to 2016 (Table S1) were provided by L. Sarabando (Baixo Vouga Forestry Association).

All calculations were discounted to present values (2016) in euros using a 4% discount factor, which is the value currently recommended by the European Commission for the cost-benefit assessment of publicly funded projects (Sartori et al. 2014). Because calculations were based on uncertain assumptions, sensitivity analyses were performed for the stumpage price (-20% versus +20%) and for the discount rate (3% versus 5%).

2.2. Economic benefit of *A. nitens* in Portugal

The economic benefit resulting from biological control was assessed for the 1996-2016 period by comparing current losses (with biological control, Scenario 0), estimated in Section 2.1, with losses that would have occurred in the absence of *A. nitens*. Considering a hypothetical situation without parasitism, total yield loss by *G. platensis* could have occurred. This assumption is based on observations of total wood loss in Portugal, when parasitism rates are extremely low (Valente et al. 2004; Reis et al. 2012), and in South Africa, when the snout beetle was free from biological control (Tooke 1955). Even though 100% wood production loss would be expected without *A. nitens* or other control methods, a more conservative value of 75% was assumed in our analysis.

Three scenarios without parasitism by *A. nitens* were considered. In Scenario 1, forest owners were assumed to have replaced *E. globulus* with eucalypt species less susceptible to *G. platensis*. This replacement would only have been possible if adequate alternatives were available, but species with wood quality for pulping similar to *E. globulus* and simultaneously well adapted to Portuguese environmental conditions would be hard to find, if they exist at all. Still, examples of species that are generally less attacked by the snout beetle and could be used for this purpose are mentioned by Cordero-Rivera and Santolamazza-Carbone (2000). *Eucalyptus globulus* plantations would then be replaced at a rate of 25 thousand ha per year.

This rate was estimated from data referring to new plantations of *Eucalyptus* spp., conducted by the pulp and paper companies operating in Portugal. Between 2010 and 2015, these companies managed 154 861 ha and planted 4 772 ha per year, on average (CELPA 2016). The same rate of planting was then applied to 811 943 ha, the total area of eucalypt plantations in Portugal, according to the latest national forest inventory (ICNF, 2013). Replacement of *E. globulus* stands would only have started in 2000, so that a four year time interval would have allowed for the identification of alternative tree species and for the production of the plants needed. New plantations were not considered as an additional investment, but rather as the standard practice of replacing *E. globulus* plantations at cutting age. For simplification, new plantations were assumed to have the same productivity and market value as *E. globulus*, even though wood from resistant eucalypts would predictably have a lower market value.

In Scenario 2, insecticides would be used to control *G. platensis* populations. It seems likely that only part of the area affected by the snout beetle would then be treated, mostly due to legal and forest certification restrictions to pesticide use (e.g. distance to water sources). Insecticides were therefore assumed to have been used annually in half of the area attacked. Insecticide applications would have started in 2000, so that adequate insecticides could be identified and legally authorised. Based on the results of efficacy studies performed for several insecticides under laboratory and field conditions (Pérez Otero et al. 2003; Santolamazza-Carbone and Ana-Magán 2004; Loch 2005; Echeverri-Molina and Santolamazza-Carbone 2010), chemical treatments were assumed to be 100% effective in controlling the snout beetle. A single insecticide application would prevent wood losses in the treated areas during one year, as shown by Loch (2005) for alpha-cypermethrin treatments in Western Australia. The cost of treating one hectare with insecticide (one application per year) was considered to be 45 euros, based on current average market prices (C. Valente, personal observation).

In Scenario 3, no replacement of the planted *Eucalyptus* species would take place and insecticides would not be applied, implying that replacement wood would have to be imported to supply the pulp and paper industry. Because in the study area eucalypts are normally harvested when plantations reach 12 years, the amount of wood that would have to be imported in a given year y (IMP_y ; $m^3 \text{ob. year}^{-1}$) was

assessed using Eq. (3), where WVL ($m^3 \text{ob. year}^{-1}$) is wood loss due to *G. platensis* in the previous years:

$$IMP_y = \sum_{i=1}^{12} \frac{1}{12} \cdot WVL_{y-i} \quad \text{Eq. (3)}$$

Annual economic losses in this scenario were calculated by multiplying the wood volume imported each year by the corresponding price of imported wood. Annual prices of wood imports between 1997 and 2016 (Table S1) were provided by F. Goes (CELPA, Portuguese Paper Industry Association).

Due to uncertainty linked to some parameters, sensitivity analyses were performed for all scenarios for: i) percentage of wood loss caused by *G. platensis* in the absence of parasitism (50% versus 100%); ii) wood price (-20% versus +20%); and iii) discount rate (3% versus 5%).

2.3. Cost-benefit analysis of the CBC programme with *A. nitens* in Portugal

A *post hoc* analysis was performed to determine the benefit-cost ratio of the biological control programme started in 1997, which aimed to accelerate *A. nitens* establishment in Portugal. Costs and benefits were discounted to present (2016) values in euros using a 4% discount rate. Programme costs were assessed through the sum of the expenses involved in the acquisition, mass rearing, releasing, and monitoring of *A. nitens*, namely costs with personnel, parasitoid purchase, facilities and equipment, maintenance, electricity, water, materials, and travel expenses (Table S2). These costs were obtained by consulting internal documentation available at RAIZ (Research Institute for Forestry and Paper), the institution that carried out most of the programme activities, in collaboration with other organisations (see Valente et al. 2004).

If the mentioned biological control programme had not been implemented, *A. nitens* would still have spread naturally from Spain, where it was first released in 1994 (Pérez Otero et al. 2003). Yet this would have resulted in a delay in the establishment of the parasitoid between one and three years, assuming dispersal rates observed in other regions (Tooke 1955; Pinet 1986). To assess the benefits of releasing *A. nitens* in the study area in order to anticipate its establishment, three alternative scenarios without releases were considered, assuming that the outcome of biological control would have been delayed by one, two, or three years. Economic

losses were estimated as in Section 2.1. Sensitivity analyses were performed for: i) percentage of wood loss caused by *G. platensis* in the absence of parasitism (50% versus 100%); ii) wood price (-20% versus +20%); and iii) discount rate (3% versus 5%).

3. Results

3.1. Economic impact of *G. platensis* in Portugal

Results of the survey conducted between 2011 and 2014 showed that 46% of the area planted with eucalypts in Portugal was attacked by *G. platensis*, with 17% having Low defoliation, 17% having Moderate defoliation, and 12% having High to Very high defoliation (Table S3). High or Very high defoliation levels were detected in 14 of the 28 NUTS3 regions, all located in the northern half of the country. Despite the partial success attained with CBC with *A. nitens*, up to about 1M m³ob of tradeable eucalypt wood have been lost annually due to *G. platensis* (Table S3). This wood volume corresponds to an economic loss of about 27M euros per year, considering stumpage wood price in 2016 (26 euros.m⁻³ob). For the entire study period (1996-2016) losses would have accumulated to 648M euros, at a 4% discount rate relative to the base year 2016 (Table S4; Table 2.1). By varying the parameters used in the calculations (yield reduction by *G. platensis* without parasitism, wood price, and discount rate), estimated total losses would have ranged from 518M to 777M euros (Table 2.1).

3.2. Economic benefit of *A. nitens* in Portugal

Economic losses calculated annually for the study period (1996-2016), considering the three scenarios without *A. nitens*, are shown in Table 2.1. Accumulated losses over 20 years would have reached 2 546M euros in Scenario 1, 2 451M euros in Scenario 2, and 7 164M euros in Scenario 3. By subtracting the economic loss in the real situation (with *A. nitens*; 648M euros) from the minimum loss value for the three scenarios without *A. nitens* (2 451M euros in Scenario 2), a benefit of at least 1 803M euros would have resulted from biological control. By varying the parameters used in sensitivity analyses, economic losses without *A. nitens* would have ranged between 1

354 and 3 739M euros in Scenario 1, between 1 767M and 3 683M euros in Scenario 2, and between 4 776M and 9 552M euros in Scenario 3.

3.3. Cost-benefit analysis of the CBC programme with *A. nitens* in Portugal

The cost of the CBC programme, carried out from 1997 to 2003, was estimated at ca. 1.1M euros at present values (details in supplementary Table S2). Assuming that biological control of *G. platensis* would have been delayed by one to three years if the programme had not been executed, the net benefit resulting from parasitoid releases would range from 75M to 389M euros for a delay of one and three-years, respectively (Table 2.2). Benefit-cost ratios would be 67, 190, and 347 for one, two, or three years without successful biological control by *A. nitens*, respectively. By varying the parameters in sensitivity analyses, benefit-cost ratios ranged from 39 to 489 (Table 2.2).

Table 2.1 Economic value of wood lost due to *Gonipterus platensis* in Continental Portugal, between 1996 and 2016, in the real situation with parasitism by *Anaphes nitens* (Scenario 0) and three hypothetical scenarios without biological control (Scenarios 1-3). The parameters varied in the sensitivity analyses were the percentage of yield reduction by *G. platensis* in the absence of biological control (50% and 100%), wood price (-20% and +20%; applied to import prices in Scenario 3 and to stumpage prices in the remaining calculations), and discount rate (3% and 5%).

Scenario	Base scenario (million euros) ^a	Sensitivity analyses (million euros)					
		Yield reduction by <i>G. platensis</i>		Wood price		Discount rate	
		50%	100%	-20%	+20%	3%	5%
0: Real situation ^b	648	642	654	518	777	592	710
1: Eucalypt replacement ^c	2 546	1 354	3 739	2 145	3 218	2 298	2 825
2: Insecticide application ^d	2 451	1 767	3 136	2 041	3 683	2 242	2 685
3: Wood imports ^e	7 164	4 776	9 552	5 730	8 603	6 732	7 632

^a Base scenario assuming 75% yield reduction by *G. platensis*, wood prices at annual stumpage prices (Scenarios 0, 1, and 2) or import prices (Scenario 3), and values discounted at 4% relative to the base year 2016.

^b Scenario 0- Current circumstances, with *A. nitens* present in Portugal since 1997.

^c Scenario 1- *Eucalyptus globulus* plantations replaced by resistant eucalypts from 2000 onward, at a rate of 25 thousand ha.year⁻¹.

^d Scenario 2- Insecticides applied once a year, from 2000 onward, in 50% of the area affected by *Gonipterus platensis*.

^e Scenario 3- Wood lost replaced by imported wood and losses valued at import prices.

Table 2.2 Costs, benefits, and benefit-cost ratios of the biological control programme with *Anaphes nitens* in Continental Portugal versus three scenarios of no release with varying delay times in parasitoid establishment (one, two, and three years). The parameters varied in the sensitivity analyses were the percentage of yield reduction by *Gonipterus platensis* in the absence of biological control (50% and 100%), wood price (-20% and +20%; applied to import prices in Scenario 3 and to stumpage prices in the remaining calculations), and discount rate (3% and 5%).

	Delay in <i>A. nitens</i> establishment	Base scenario ^a	Sensitivity analyses					
			Yield reduction by <i>G. platensis</i>		Wood price		Discount rate	
			50%	100%	-20%	+20%	3%	5%
Costs (million euros)	n.a.	1.1	1.1	1.1	1.1	1.1	1.0	1.3
Benefits (million euros)	1 year	75.1	43.8	106.3	60.1	90.1	63.1	89.2
	2 years	213.2	125.9	300.6	170.6	255.9	180.3	251.8
	3 years	389.1	229.9	549.4	311.3	466.9	331.0	456.7
Benefit-cost ratio	1 year	67	39	94	54	80	65	68
	2 years	190	112	268	152	228	189	191
	3 years	347	205	489	277	416	346	347

^a Base scenario assuming 75% yield reduction by *G. platensis*, wood prices at annual stumpage prices (Scenarios 0, 1, and 2) or import prices (Scenario 3), and values discounted at 4% relative to the base year 2016.

Table 2.3 Overview of expected impacts from defoliation by *Gonipterus platensis*.

Type of impact	Impact on services	References
Provisioning ecosystem services	Reduced pulpwood yield.	Reis et al. (2012), present study
	Impacts on honey production, because eucalypts are major sources of pollen and nectar for honeybees.	Daners and Tellería (1998), Feás et al. (2010)
	Reduced aesthetic value of eucalypts used as ornamental trees (e.g. parks and roadsides).	Paine et al. (2015)
	Increased management costs and environmental risks due to the use of insecticides to control the pest.	Pimentel et al. (1992), Sexton et al. (2007)
Socio-economic activities	Impact on the Portuguese economy (the pulp and paper industry contributes with 4.4% to the gross domestic product and represents 5% of the country's exports, valued at ca. 2 500M euros in 2015).	CELPA (2016), INE (2016)
	Reduction in employment (forestry and logging activities ^a are estimated to generate 13 500 direct jobs, particularly in rural areas; the manufacture of paper and paper products ^b assures about 17 800 jobs, 3 000 of which directly by the Portuguese pulp and paper industry).	CELPA (2016), EUROSTAT (2017).
	Decreased economic return leads forest owners to reduce forest management, leading to changes in land use and value.	Kenis and Branco (2010)
Non-provisioning services of ecosystem	Decreased carbon sequestration.	Pinkard et al. (2014)
	Decreased water retention and increased nutrient leaching.	Fernández et al. (2006), Lovett et al. (2002)
	Lower ability of weakened eucalypt plantations to compete with invasive plant species, such as wattles (<i>Acacia</i> spp.), leading to severe changes in ecosystem structure and functioning.	Fernández et al. (2006), Lorenzo et al. (2010)

^a NACE A02, according to the European Classification of Economic Activities (EUROSTAT, 2008).

^b NACE A17, according to the European Classification of Economic Activities (EUROSTAT, 2008).

4. Discussion

According to the present assessment, defoliation by *G. platensis* resulted in wood losses of 648M euros in the study area over the past 20 years. The most severe attacks occurred in the north of the country, in cool and mountainous regions, as suggested by previous studies (Valente et al. 2004; Reis et al. 2012). Such economic losses happened in spite of partial success of biological control by *A. nitens*. Without parasitism, losses would predictably have ranged from 2 451M euros, in a scenario where *G. platensis* populations were controlled with insecticides, to almost 7 200M euros, if wood losses were offset by imported wood. Therefore, the benefit of biological control with *A. nitens* in the study area during the last two decades amounted to at least 1 803M euros (2 451M - 648M euros). By varying the parameters in the sensitivity analyses, economic losses without biological control would have ranged from 1 354M to 9 552M euros, for Scenarios 2 and 3, respectively. These extreme values were obtained by varying the percentage of wood loss (50 and 100%) caused by *G. platensis*. Regardless of the parameters used in the sensitivity analyses, partial biological control under the current circumstances (Scenario 0) is by far the most favourable scenario. By varying the parameters in the analyses, both eucalypt replacement (Scenario 1) and insecticide application (Scenario 2) would account for economic losses about two to four times higher than with *A. nitens*. Wood imports (Scenario 3) are the worst outcome not only in a straightforward cost-benefit analysis but also considering important negative impacts on employment generated by forestry activities (see discussion below).

While Scenarios 1 and 2 produced very similar economic outcomes, it is interesting to note that, for a yield reduction by *G. platensis* of 50%, eucalypt replacement would be preferable to insecticide application, whereas for 100% of yield reduction the more immediate effect of insecticides would be more cost-effective. Variations in the valuation of wood also lead to differences in the outcomes of Scenarios 1 and 2. For a higher (+20%) wood price, the fact that only half of the affected area could be treated with insecticides leads to higher economic losses, and eucalypt replacement would be the best management option in the long run.

Despite our attempt to use realistic scenarios, it's doubtful that the three scenarios without parasitism by *A. nitens* considered here would be sustainable. In Scenario 1, eucalypt species both resistant to *G. platensis* and endowed with characteristics

similar to *E. globulus* would have to be available. Due to the favourable adaptation of *E. globulus* to the Portuguese environmental conditions and to the high quality of this species' wood for pulp production, such a replacement would be difficult. In cooler northern regions of Portugal and Spain where severe defoliation by *G. platensis* occurs regularly, *Eucalyptus nitens* Maiden has been planted as an alternative to *E. globulus* (Pérez-Cruzado et al. 2011). However, *E. nitens* has important disadvantages when compared to *E. globulus*, such as poor coppicing ability (Little et al. 2002) and lower pulpwood quality (Kibblewhite et al. 2000). Regarding Scenario 2, the use of insecticides in forests poses several disadvantages in comparison to biological control, since ecological, environmental, and economic impacts may occur. Two commercial insecticides, Calypso (active ingredient thiacloprid) and Epik (active ingredient acetamiprid), are currently authorised in Portugal against *G. platensis* (ICNF 2015). Epik is also authorised in Spain (MAPAMA, 2017). In Portugal, chemical control has been carried out with Calypso since 2011 and with Epik since 2012, with good results (C. Valente, unpublished data). Still, the use of insecticides has limitations and risks that were not accounted for in this study. Among others, non-target organisms may be affected, the target organism may develop resistance, there is the risk of soil and water contamination, and repeated applications are often necessary. Additionally, insecticide use is constrained by legal and forest certification restrictions and public concern over pesticide use is an important issue (Pimentel et al. 1992; Jetter and Paine 2004; Sexton et al. 2007). Due to such difficulties, repeatedly treating half of the area affected by the snout beetle, as predicted in Scenario 2, might have been impracticable. As for Scenario 3, it is possible that the large amount of wood needed would not be readily available for import from external markets. Furthermore, the higher costs of wood in this scenario (compared to the costs of wood produced locally) would reduce the market competitiveness of the pulp and paper companies in Portugal. Based on data gathered from statistical reports published by the Portuguese Paper Industry Association (CELPA 2007, 2016), ca. 22.8M m³ of eucalypt wood were imported between 1997 and 2015, 56% of which in the last five years. Although damage by *G. platensis* might not be the sole reason for the sharp increase in imports, it is likely a major driver, as our estimates of wood loss due to defoliation for the same period equal 75% of these imports (17.4M m³). A more realistic scenario should assume the simultaneous implementation of the three options identified: replacement of *E. globulus* by less susceptible species, use

of insecticides, and wood import, as in fact has happened in Portugal. However, the analysis of such scenario would be very complex and higher levels of uncertainty would be introduced.

The biological control programme planned to accelerate the establishment of *A. nitens* in Portugal had a positive return on investment. Its minimum benefit-cost ratio was estimated at 67, when the benefits of releasing *A. nitens* were considered to have occurred in one year only, and accrued to 190 or 347 if benefits for two or three years, respectively, were taken into account. The most extreme values of benefit-cost ratios were obtained in sensitivity analyses, by varying yield reduction (50 or 100%) due to *G. platensis* in the absence of parasitism. Unlike the trade-offs revealed by sensitivity analyses for other pest management practices (Table 2.1), biological control lead to benefits that increase consistently with the degree of anticipation of its effects, regardless of variations in pest defoliation, wood price, or discount rate (Table 2.2). The time delay in biological control of one to three years, predicted in our study for a situation without a CBC programme, is based on observations by Tooke (1955) and by Pinet (1986). Tooke (1955) reported limited dispersion of *A. nitens* during the first two seasons after its introduction in South Africa, but recorded a fast spreading rate ($> 100 \text{ km}\cdot\text{year}^{-1}$) once the parasitoid populations became well established. Nevertheless, a spreading delay of three years may be underestimated, as a longer period might have been needed for the parasitoid to spread naturally from Galicia (Spain) to central/southern Portugal, covering ca. 300-400 km. In fact, Pinet (1986) recorded a slow dispersal of *A. nitens* in France, after its introduction in Italy, near the border between the two countries. In three years (1978-1981), *A. nitens* had spread only about 40 Km in France, and in 1981 the parasitoid had to be released in several locations that remained without parasitism (Pinet 1986).

The benefit-cost ratios obtained in the present study are positive, similarly to what was found for other CBC programmes that were evaluated economically (Naranjo et al., 2015). The ratios found in our study are conservative, as only 75% yield loss caused by *G. platensis* was assumed, instead of the more likely 100% loss. Furthermore, the cost-benefit analyses performed here included post-release monitoring costs between 2001 and 2003, which were valued at about 20% of the total costs. As a result, the costs directly contributing to the benefits are

overestimated in the analyses. Inversely, by using a well-known natural enemy, the costs of this programme were lower than if a new CBC agent had to be identified in the pest's native range. This is likely to be the case for other parasitoids that have recently been evaluated as alternative CBC agents, such as the Tasmanian *Anaphes inexpectatus* Huber and Prinsloo (Valente et al. 2017a,b) and *Anaphes tasmaniae* Huber and Prinsloo (Ide et al. 2013).

Our results underestimate the impact of both the damage caused by *G. platensis* and the benefit from *A. nitens*, because calculations were based exclusively on their impact on wood production. Even though pulpwood is regarded as the key provisioning service provided by eucalypt plantations, other ecosystem services and socio-economic benefits are also provided (Branco et al. 2015). Other possible impacts resulting from *G. platensis* defoliation, which are summarised in Table 2.3, can be as important as those on wood production itself (Holmes et al. 2009). Socio-economic impacts in particular may be of great relevance, since the activities related to the pulp and paper production assume an important role for the economy and the social sector, mainly regarding employment. In Portugal, the manufacture of paper and paper products assures 17 800 direct jobs and forestry and logging activities are estimated to generate 13 500 direct jobs (EUROSTAT 2017) (see socio-economic activities in Table 2.3). Indirectly, this impact would extend to hundreds of thousands of small land owners that depend on forestry activities as a supplementary source of income, mostly in underprivileged rural areas (Sarmiento and Dores 2013). Even if unemployment resulting from *G. platensis* attacks would reallocate to other activities, the negative impact would be not negligible, particularly in the forestry sector.

Our economic analysis highlights the importance of prompting control strategies immediately following invasion, as anticipating control by even a single year may have a positive economic impact. This result should encourage decision makers to rapidly implement effective control against important invasive alien species. Even considering some unfavourable assumptions, as we did in the sensitivity analyses, the biological control programme remains cost-effective. Our results further suggest that even partially successful CBC programmes may provide economic benefit. As shown by McFayden (2008) for two programmes against the weeds *Lantana camara* L. and *Rubus fruticosus* L. in Australia, economic benefits can be attained even from CBC projects ultimately considered failures. Positive outcomes from

apparent failures, or low success actions, can occur when the target species has high economic impact, as even a small reduction in losses is economically relevant. Gathering the information necessary to conduct this study proved to be a laborious task that required intensive effort, but our findings emphasize the importance of measuring the success of CBC programmes on the basis of their economic impact, rather than by merely quantifying technical and/or biological parameters, such as parasitism rates. Economic evaluations also provide useful information that can help decision makers and stakeholders select the most appropriate pest management strategies.

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Supplementary material

Table S1 *Eucalyptus* wood prices from 1997 to 2016 (stumpage price is the price before harvesting and transportation to the mill; ob= over bark). Sources: L. Sarabando, Baixo Vouga Forestry Association (Stumpage prices) and F. Goes, CELPA (Import prices).

Year	Stumpage price (euros.m ⁻³ ob)	Import price (euros.m ⁻³ ob)
1997	23	76
1998	23	73
1999	23	66
2000	23	59
2001	23	55
2002	23	62
2003	23	87
2004	23	90
2005	23	42
2006	21	69
2007	26	80
2008	30	74
2009	25	66
2010	26	78
2011	30	75
2012	25	77
2013	25	80
2014	25	74
2015	26	87
2016	26	84

Table S2 Costs of the biological control programme with *Anaphes nitens* in Continental Portugal, between 1997 and 2003. Economic values shown are nominal and discounted with a 4% discount rate relative to the base year 2016.

Year	Year number ^a	Discount factor	Nominal costs (euros)				Discounted costs (euros)	
			Parasitoid importation from Spain	Rearing and release	Monitoring and research	Total	Total	
1997	-19	2.11	6 090	4 000	14 000	24 090	50 754	
1998	-18	2.03	12 120	161 067	14 000	187 187	379 206	
1999	-17	1.95	12 030	92 067	14 000	118 097	230 041	
2000	-16	1.87	14 425	95 067	14 000	123 492	231 298	
2001	-15	1.80	-	-	44 400	44 400	79 962	
2002	-14	1.73	-	-	44 400	44 400	76 886	
2003	-13	1.67	-	-	44 400	44 400	73 929	
TOTAL			44 665	352 200	189 200	586 065	1 122 076	

^a Relative to 2016.

Table S3 Annual impact of *Gonipterus platensis* in Continental Portugal for NUTS3 (Nomenclature of Territorial Units for Statistics, version 2010) regions, according to the results from a survey conducted between 2011 and 2014 (present study); ob= over bark.

NUTS3	Eucalypt area by defoliation level (ha)					Wood loss	
	No damage	Low	Moderate	High	Very high	(m ³ ob.year ⁻¹)	(million euros)
Alentejo Central	24 589	0	0	0	0	0	0.0
Alentejo Litoral	58 330	0	0	0	0	0	0.0
Algarve	24 922	0	272	0	0	487	0.0
Alto Alentejo	42 051	0	0	0	0	0	0.0
Alto Trás-os-Montes	147	5 276	2 053	572	0	5 055	0.1
Ave	3 661	12 772	2 741	50	0	26 105	0.7
Baixo Alentejo	12 229	0	0	0	0	0	0.0
Baixo Mondego	43 159	1 101	0	0	0	849	0.0
Baixo Vouga	4 327	25 983	17 957	13 325	0	158 161	4.1
Beira Interior Norte	0	153	2 161	79	0	2 863	0.1
Beira Interior Sul	16 737	10 461	15 721	12 071	0	44 359	1.2
Cávado	1 428	5 660	4 432	249	1 039	51 190	1.3
Cova da Beira	33	1 278	2 937	1 660	168	13 317	0.3
Dão-Lafões	5 853	14 514	16 435	10 925	3 581	164 542	4.3
Douro	13	1 727	1 789	1 152	0	5 835	0.2
Entre Douro e Vouga	0	3 974	16 139	7 952	8 201	201 770	5.2
Grande Lisboa	5 549	0	0	0	0	0	0.0
Grande Porto	1 812	11 868	1 298	12	0	18 017	0.5
Lezíria do Tejo	67 456	0	0	0	0	0	0.0
Médio Tejo	49 496	603	0	0	0	311	0.0
Minho-Lima	1 000	5 455	10 253	5 450	222	102 651	2.7
Oeste	36 100	2 073	486	0	0	2 496	0.1
Península de Setúbal	10,993	0	0	0	0	0	0.0
Pinhal Interior Norte	4 998	15 890	18 635	20 977	250	151 056	3.9
Pinhal Interior Sul	11 864	8 373	4 169	0	0	10,681	0.3
Pinhal Litoral	18 514	0	0	0	0	0	0.0
Serra da Estrela	0	842	0	0	0	473	0.0
Tâmega	1 282	12 768	12 153	6 722	341	82 351	2.1
Total	446 543	140 771	129 631	81 196	13 802	1 042 569	27.1
% of Total	54	17	17	10	2		

Table S4 Impact of *Gonipterus platensis* in Continental Portugal, between 1996 and 2016, on *Eucalyptus globulus* yield (wood volume and economic value of wood; ob= over bark). Economic values shown are nominal and discounted with a 4% discount rate relative to the base year 2016.

Year	Wood lost	Economic value of wood lost	
	(million m ³ ob)	Nominal (million euros)	Discounted (million euros)
1996	0.00	0.0	0.0
1997	0.38	8.7	18.4
1998	0.40	9.2	18.6
1999	0.68	15.6	30.3
2000	0.92	21.2	39.7
2001	0.93	21.5	38.7
2002	0.93	21.5	37.2
2003	0.93	21.5	35.8
2004	0.93	21.5	34.4
2005	1.01	23.2	35.7
2006	1.01	21.2	31.4
2007	1.01	26.2	37.3
2008	1.01	30.3	41.4
2009	1.01	25.2	33.2
2010	1.04	27.1	34.3
2011	1.04	31.3	38.1
2012	1.04	26.1	30.5
2013	1.04	26.1	29.3
2014	1.04	26.1	28.2
2015	1.04	27.1	28.2
2016	1.04	27.1	27.1
Total	18.5	458	648

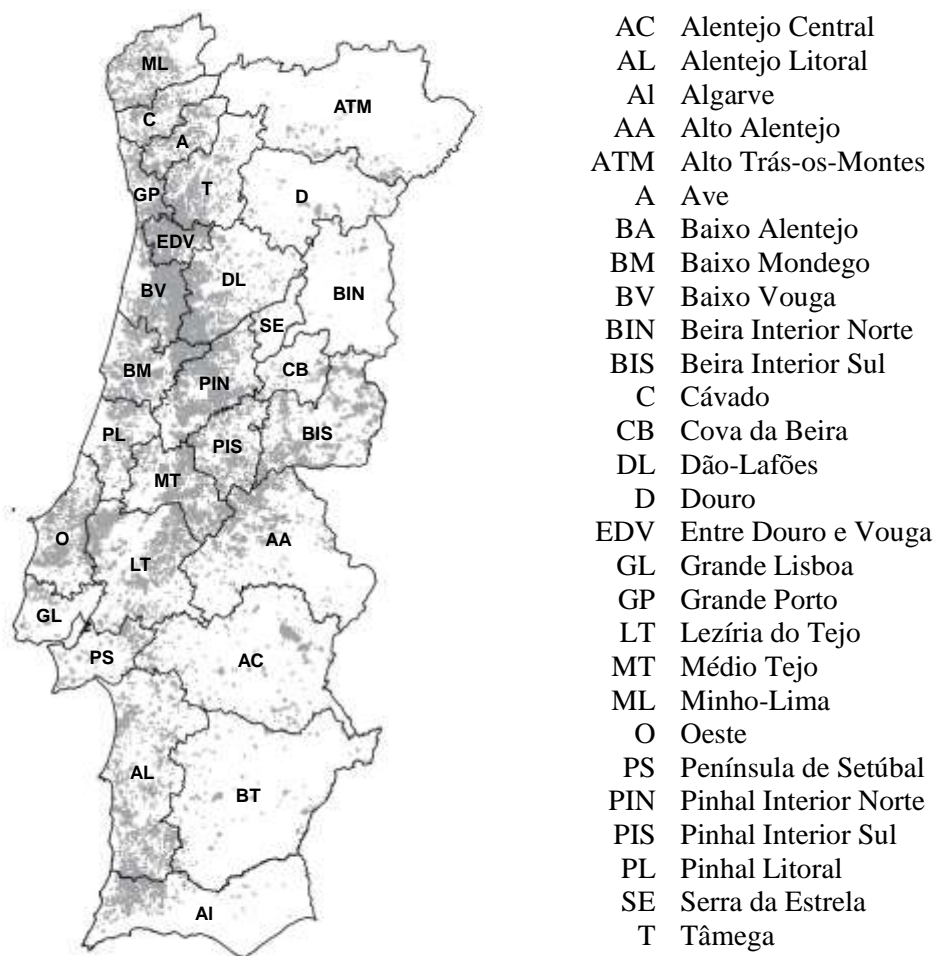


Fig. S1 Territorial units NUTS3 (Nomenclature of Territorial Units for Statistics, version 2010) and eucalypt distribution (shaded area) in Continental Portugal in 2005. Sources: ICNF (2013) and EUROSTAT (2016).

Appendix 1 Procedures used in Section 2.1.1 to assess the area affected by *Gonipterus platensis* in Portugal during its spreading phase (1996-2003).

The first sampling point was located at the edge of the snout beetle's known distribution from the previous year, where the insect was assumed to be present. From there, observations were made every 4 km in the most likely direction of dispersal, typically south and east, until neither signs of damage nor insects were detected. At each sampling point, the canopy of every eucalypt in the observer's field of vision was carefully examined with binoculars, in order to detect *G. platensis*. Absence of *G. platensis* in a given sampling point was confirmed by checking two more points with eucalypts located in the same direction. Once a point of no detection was reached, the survey would resume in a new direction from the last sampling point where the snout beetle was detected. In order to construct a comprehensive map, presence or absence of *G. platensis* was assigned to "Freguesia", the smallest Portuguese administrative territorial unit.

Appendix 2 Procedures used in Section 2.1.2 to assess the area affected by *Gonipterus platensis* in Portugal between 2011 and 2014.

Defoliation data was collected annually (between June and October) after the annual defoliation peak by *G. platensis*, which normally occurs in May. Only plantations older than 1.5 years were evaluated in order to assure that trees had adult foliage, which is in general more susceptible to *Gonipterus* attack than juvenile foliage (Tooke, 1955). Defoliation was categorised into the following five damage categories, based on the leaf area loss in the upper third of each tree canopy: 1) No damage (no defoliation); 2) Low (1-20% defoliation); 3) Moderate (21-60% defoliation); 4) High (61-90% defoliation); and 5) Very high (> 90% defoliation). A total of ca. 1 400 plantations were surveyed, ranging from 1 ha to about 3 000 ha. Depending on plantation size and heterogeneity (defoliation, topography, stand age, and eucalypt provenance or clone), 1 to 30 sampling points were inspected per plantation. At each sampling point, the trees in the observer's field of vision were inspected with binoculars and overall defoliation, corresponding to the most frequent attack level observed, was estimated. Annual geographical layers produced on the four years of sampling were overlapped using QGIS 2.2.0 software. Plantation areas were broken down into single-part polygons and the highest attack level recorded during the four-year period was assigned to each polygon. Plantations were then grouped into 28 territorial units (NUTS3, Nomenclature of Territorial Units for Statistics, version 2010; Fig. S1) (EUROSTAT, 2016) and the total area per defoliation level and NUTS3 region was calculated.

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Anaphes inexpectatus parasitising an egg capsule and adult of *Gonipterus platensis* (photo by C. Gonçalves).

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Pre-selection and biological potential of the egg parasitoid *Anaphes inexpectatus* for the control of the *Eucalyptus* snout beetle, *Gonipterus platensis*

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Abstract

The *Eucalyptus* snout beetle, *Gonipterus platensis* (Marelli), causes severe damage to eucalypt plantations in several countries, despite the presence of the parasitoid *Anaphes nitens* (Girault). Climate and/or host-parasitoid mismatch may explain *A. nitens* shortcomings in some areas in Portugal, Spain, Chile, South Africa, or Australia. Because additional parasitoids may be needed to achieve reliable control of this pest, *Anaphes inexpectatus* Huber and Prinsloo, retrieved from field surveys conducted in Tasmania (the pest's native habitat), was selected for pre-release studies in Portugal. Life history traits of *A. inexpectatus* and *A. nitens* were compared at six temperatures (5, 10, 15, 20, 25, and 30 °C), including development times, thermal constants, viability, parasitism, and behaviour. Temperatures ranging from 10 to 20 °C were adequate for development, while at 25 and 30 °C deleterious effects of temperature were detected, particularly in *A. nitens*. Development thresholds were similar for *A. inexpectatus* and *A. nitens* (6.0 and 5.4 °C, respectively), but *A. nitens* needed 313 degree-days to complete development, while *A. inexpectatus* needed 263 degree-days. Globally, *A. nitens* produced more progeny, parasitised more eggs, and lived longer than *A. inexpectatus*. Net reproductive rates were higher for *A. inexpectatus* at lower temperatures (10 and 15 °C), and higher for *A. nitens* at moderate temperatures (20 and 25 °C). In addition, *A. inexpectatus* evidenced higher tolerance to the highest temperature tested (30 °C). *Anaphes inexpectatus* is likely to establish under field conditions and may enhance parasitism of *G. platensis*.

Keywords

Anaphes nitens, *Eucalyptus* weevil, biological control, *Eucalyptus globulus*, thermal biology.

1. Introduction

The *Eucalyptus* snout beetle, *Gonipterus platensis* (Marelli) (Coleoptera: Curculionidae), is one of the most destructive eucalypt pests worldwide. Previously referred to as *G. scutellatus*, this designation encompasses a group of cryptic species (Mapondera et al. 2012). *Gonipterus platensis* is native to Tasmania, and is the most widely distributed *Gonipterus* species outside Australia. Its continued activity leads to loss of apical dominance, stunted growth, and wood losses (Tooke 1955; Loch 2006; Reis et al. 2012). Biological control with the egg parasitoid *Anaphes nitens* (Girault) (Hymenoptera: Mymaridae) has been the most successful strategy against *Gonipterus* spp. (Tooke 1955; Huber 1986; Mansilla Vázquez et al. 1998; Hanks et al. 2000; Valente et al. 2004; SAG 2005).

In Europe's Mediterranean basin, *Gonipterus* spp. may display up to three generations per year, with most damage being caused by the spring generation, usually between February and April (Cordero-Rivera et al. 1999; Santolamazza-Carbone et al. 2006; Branco et al. 2016). In Portugal, *G. platensis* established in 1995 and rapidly became a key pest of the widely planted *Eucalyptus globulus* Labill., with both larvae and adults feeding on newly flushed leaves, shoots, and buds. Shortly after *G. platensis* arrival, a biological control programme using *A. nitens* was established (Valente et al. 2004). Despite the success achieved with *A. nitens* throughout most of the country, this parasitoid has failed to provide satisfactory control of *G. platensis* at altitudes above 400-450 m in central and northern regions. Parasitism rates by *A. nitens* during peak egg laying periods in late winter/early spring are negatively correlated with altitude. Below altitudes of 400 m, mean parasitism rates range from 70 to 95% during April, while above 600-700 m parasitism ranges between 0 and 25% (Valente et al. 2004; Reis et al. 2012). Erratic control of *G. platensis* by *A. nitens* has been found to occur in other regions in South Africa (Tooke 1955; Tribe 2005), Spain (Cordero-Rivera et al. 1999), southwestern Australia (Loch 2008), and Chile (Gumovsky et al. 2015). A widely accepted

explanation is that, whenever foliage flushing by eucalypt trees is inhibited, as occurs during winter periods due to low temperature, *G. platensis* females are deprived of adequate oviposition sites (Tooke 1955; Tribe 2005; Loch 2008). This in turn results in long periods of low host availability for *A. nitens*. In late winter/early spring, *G. platensis* numbers rise rapidly but the surviving parasitoid population is unable to respond in adequate numbers. Although late spring parasitism rates are often over 90%, the larvae escaping parasitism early in the season have already caused substantial damage to eucalypt trees (Tooke 1955; Cordero-Rivera et al. 1999; Tribe 2005; Loch 2008; Reis et al. 2012). Average maximum temperatures during winter months (MaxTw) below a threshold temperature of 10 °C resulted in low parasitism rates of *G. platensis* by *A. nitens* during late winter (10.1%), while MaxTw above 11.5 °C resulted in 70.9% parasitism (Reis et al. 2012). Parasitoid performance at temperatures around 10 °C should therefore be crucial when estimating the potential of natural enemies against *G. platensis*. In Portugal, maximum temperatures in summer months are frequently above 25 °C, even at altitudes above 400 m (AEMET and IPM 2011). Therefore, parasitoid tolerance to high temperature will likely contribute to successful establishment. This is particularly important for the immature life stages, as adults can move to more favourable micro-environments (Collins and Grafius 1986; Hance et al. 2007).

Climate is likely the most important limiting factor to insect distribution (Huffaker et al. 1976), and that applies to both pests and natural enemies (Reineke and Thiéry 2016). Temperature in particular plays a central role in insect development and has been extensively reviewed (Laudien 1973; Taylor 1981). The temperature below which an insect can survive but not develop is known as the threshold temperature (Laudien 1973; Lamb 1992). Above the threshold, development rate increases up to an optimal value above which development is reduced due to inhibition or injury (Laudien 1973; Campbell et al. 1974; Taylor 1981; Trudgill et al. 2005). Other insect life history traits are known to depend on temperature, such as life span or fecundity (Laudien 1973). Because each insect species has its own development rate with respect to temperature, even small differences in environmental conditions, such as happens in altitudinal gradients, can have a profound effect on host-parasitoid interactions (Hance et al. 2007). Another possible explanation for the lack of reliable control in some regions derives from the fact that *A. nitens* is originally from southern

Australian mainland while *G. platensis* is native to Tasmania, which may result in partial host-parasitoid mismatch (Mapondera et al. 2012).

Two other *Anaphes* species are known to parasitise *Gonipterus* spp. in Tasmania, namely *Anaphes tasmaniae* Huber and Prinsloo and *A. inexpectatus* Huber and Prinsloo (Huber and Prinsloo 1990). Efforts were conducted to introduce these two species into South Africa but they were unsuccessful due to bureaucratic reasons (Tribe 2003). Following a survey in Tasmania by C. Valente in 2008, both parasitoids were imported to Portugal for studies on their potential against *G. platensis*. Similar efforts were developed in Chile, where *A. tasmaniae* was released in 2009 and considered to have established in 2013 (Mayorga et al. 2013; SAG 2014).

While several studies have focused on the biology of *A. nitens* (Tooke 1955; Hanks et al. 2000; Santolamazza-Carbone and Cordero-Rivera 2003a, b; Santolamazza-Carbone et al. 2006, 2009), *A. inexpectatus* life history traits remain mostly unknown. *Anaphes inexpectatus* is known to differ from *A. nitens* in its smaller size and gregarious nature, with up to six parasitoids emerging from a single egg (Huber and Prinsloo 1990). The success of a natural enemy cannot be fully anticipated, but it relies on traits such as adaptability to environmental conditions, searching and reproductive ability, longevity, or synchronisation with the host (Doutt et al. 1976; Messenger et al. 1976; Stiling and Cornelissen 2005).

In this work we present the survey and studies leading to the pre-selection of *A. inexpectatus* as a biological control agent against *G. platensis*. Comparative studies on development and performance of *A. inexpectatus* and *A. nitens* over a range of temperatures are shown. The implications of the results on the potential of *A. inexpectatus* to establish and contribute to reliable control of *G. platensis* are discussed.

2. Material and Methods

2.1. Insect collection and rearing

Between 2008 and 2012, field surveys of *Gonipterus* spp. parasitoids were performed in Tasmania. Since 2010, over 5,000 egg capsules and 1,250 larvae were collected in 13 locations (Table 3.1), imported and incubated under quarantine conditions at RAIZ and Altri Florestal. *Gonipterus platensis* egg capsules or larvae

were offered to parasitoids, depending on the host life stage they emerged from. Specimen identification was performed by John Huber (Mymaridae), John LaSalle (Eulophidae, egg parasitoids), Andrew Polaszek (Aphelinidae), Alex Gumovsky (Eulophidae, larval parasitoids), Ludomír Masner (Proctotrupidae), and Bryan Cantrell (Tachinidae).

Table 3.1 Characterisation of *Anaphes inexpectatus* laboratory populations established from specimens collected in Tasmania, from *Gonipterus* spp. eggs, between 2010 and 2012.

Laboratory population	Collection site	Host plant	Latitude	Longitude	Collection date	Number of egg capsules	Number of emergences
A	Nunamara	<i>Eucalyptus</i> spp.	-41°25.089	147°15.752	Dec 2010	Not applicable ¹	4
	Tunbridge	<i>E. ovata</i>	-42°07.076	147°19.600	Dec 2010		53
B	Nunamara	<i>Eucalyptus</i> spp.	-41°25.089	147°15.752	Nov-Dec 2011	927	5
	Runnymede	<i>E. ovata</i>	-42°39.038	147°32.781	Nov-Dec 2011	522	2
	Wyena	<i>Eucalyptus</i> spp.	-41°10.293	147°16.274	Nov 2011	161	5
C	Tunbridge	<i>E. ovata</i>	-42°07.076	147°19.600	Nov 2012	1,430	497
	Whitefoord	<i>E. globulus</i>	-42°26.950	147°33.910	Nov 2012	150	13
	Woodsdale	<i>E. globulus</i>	-42°29.366	147°33.935	Nov 2012	235	7
D	Hobart	<i>E. globulus</i>	-42°52.937	147°18.023	Nov 2012	360	7
	New Norfolk	<i>E. globulus</i>	-42°47.272	147°03.743	Nov 2012	90	28
	Kingston	<i>E. globulus</i>	-42°58.294	147°16.236	Nov 2012	60	4
E	Deddington	<i>E. globulus</i>	-41°37.616	147°23.860	Nov 2012	130	27
	Grindelwald	<i>E. globulus</i>	-41°21.446	147°00.966	Nov 2012		
F	Hamilton	<i>E. ovata</i>	-42°37.846	146°54.760	Nov 2012	100 ²	39
	Hayes	<i>E. globulus</i>	-42°45.432	147°00.028	Nov 2012		
G	Runnymede	<i>E. ovata</i>	42°39.038	147°32.781	Nov 2012	450	190

¹ Number of egg capsules unavailable due to material degradation on arrival.

² Because few egg capsules were collected in these locations, they were grouped together and exact numbers per site are not available.

Among the parasitoids collected, *A. inexpectatus* readily accepted *G. platensis* eggs and was the only species to successfully establish under laboratory conditions. It was therefore pre-selected for further studies. Specimens of *A. inexpectatus* were obtained from mixed populations of seven laboratory strains (Table 3.1) and maintained at 10 °C (preliminary tests showed this was a suitable rearing temperature for this species). A population of *A. nitens* was established from

specimens collected in *E. globulus* stands in Portugal infested by *G. platensis*, and maintained at 20 °C. For both *Anaphes* species, newly emerged parasitoids were placed in glass vials (180 x 18 mm) with six to eight *G. platensis* egg capsules and honey (50% in water). Parasitoids were allowed to parasitise for up to one week, after which the parasitised egg capsules were replaced with new hosts and incubated at 15 °C.

Freshly laid *G. platensis* egg capsules (under 24 hour-old) were used in all trials. Egg capsules were obtained from a laboratory reared *G. platensis* population (kept at 20 °C), established from adults collected in *E. globulus* plantations. Forty individuals were placed in plastic boxes with perforated lids (1 L) with 3-5 *E. globulus* shoot tips, replaced twice a week.

2.2. Thermal requirements

Newly emerged parasitoids (under 24 hour-old) were used in all assays. For both *Anaphes* species, one female and one male were placed in glass vials (100 x 16 mm) with honey (50% in water) unless otherwise stated. Trials were performed under 14:10 L:D photoperiod and 80-90% RH. (5 °C), 70-90% RH (10 °C), 50-70% RH (15 and 20 °C), and 60-70% RH. (25 and 30 °C). Trials at 10 to 30 °C were performed in walk-in climatic chambers (Fitoclima 13000 EDTU), and trials at 5 °C took place in a chilling room with an air cooler (Centauro MT/I 421).

Immature development

For studies on the effect of temperature on immature development, couples of each *Anaphes* species were allowed to parasitise three *G. platensis* egg capsules for 24 hours at 20 °C, after which the adults were removed and the hosts were incubated at 5, 10, 15, 20, 25, or 30 °C (100 couples per species and temperature). Progeny emergence was checked daily to determine development times. The number and the sex of the parasitoids were recorded. Sex-ratios were calculated as the percentage of females. After adult emergence ceased, egg capsules were dissected to determine the number of *G. platensis* larvae, unviable eggs (without any signs of parasitism), and parasitised eggs. The later were detected once the pupae had started to develop (the first evidence being reddish/brownish eyes) and were further categorised as: (1)

undeveloped; (2) fully developed (adults were developed and ready to emerge but failed to do so); and (3) emerged. When no parasitised eggs were detected, females were categorised as non-parasitising.

Lower development thresholds (LDT) and sum of effective temperatures (SET) were determined as described by Honek (1996). SET is defined as the number of heat units (degree-days, DD) above LDT required to complete development. Calculations were based on development times at four constant temperatures (10, 15, 20, and 25 °C), using the linear regression: $R = aT + b$, where R is development rate (1/duration of development), a is the slope of the regression, T is temperature, and b is the intercept on the y axis. LDT and SET were calculated as $LDT = -b/a$ and $SET = 1/a$.

Adult performance and behaviour

Couples of each *Anaphes* species were allowed to parasitise three *G. platensis* egg capsules at 5, 10, 15, 20, 25, or 30 °C for the duration of the female life (40 couples per species and temperature). Female behaviour (mating, resting, searching, antennation, or oviposition) was recorded during the first hour after hosts were offered, at every three minutes, totalling 20 observations per insect. Parasitised egg capsules were replaced with fresh hosts twice per week, until the female died, and incubated at 15 °C. The emerging progeny was counted and sexed, and the egg capsules were dissected as described for the trial on immature development. Female fecundity was calculated as the sum of all emerging, undeveloped, and fully developed progeny. Female and male longevity was recorded daily to estimate longevity with food and hosts. Longevity with food and without hosts and longevity without food or hosts were further determined for 40 couples of each *Anaphes* species at the same six temperatures.

2.3. Statistical analysis

ANOVA and post-hoc LSD tests were used to determine the effect of *Anaphes* species and gender on development times (per temperature), and the effect of diet, temperature, and species on longevity (per gender). Generalised linear models (GLM) with Binomial distribution were used to determine the effect of rearing temperature and species on the proportion of fully developed progeny, emerging

progeny, and unviable eggs (trial on thermal requirements: immature development). GLM with Negative Binomial distribution were used to determine the effect of temperature and species on fecundity, number of parasitised eggs, number of emerging progeny, proportion of fully developed progeny, and progeny sex-ratio (trial on thermal requirements: adult performance and behaviour). Wald Chi-square statistic (W) and p values are presented. Models were first applied with two factors, temperature and species, and each species was treated separately whenever the interaction term was significant. Pearson correlations between female age and fecundity were calculated using Microsoft Excel 2010. Life history parameter estimates were based on female fertility and survivorship. Parasitoid survivorship (median and standard error) was estimated with Kaplan–Meier survival, for species, sex, and temperature, for the regime with honey and hosts. Net reproductive rates (R_0) were estimated as $R_0 = \sum mx.lx$, where mx is female fertility at day x (i.e. originating adult female progeny), and lx is female survivorship at day x . Generation times (T) were estimated as $T = \sum x.lx.mx/R_0$, where x is the time interval. Intrinsic rates of natural increase (r) were estimated as $r = \ln(R_0)$. Jackknife resampling method was used to estimate mean and standard error (Maia et al. 2000). Searching and reproductive behaviour (mating, antennation, and oviposition) were analysed with logistic models. ANOVAs were performed using Statistica 13. GLMs, survival analysis, and logistic models were performed using SPSS 21.

3. Results

3.1. Parasitoid collection and *A. inexpectatus* pre-selection

Four *Gonipterus* species were collected, namely *G. platensis*, *G. pulverulentus*, *G. scutellatus*, and *Gonipterus* sp. n. 1 *sensu* Mapondera et al. (2012) (R. Oberprieler, pers. com.), although host-parasitoid matches were not established.

The most abundant egg parasitoids were *A. tasmaniae* and *A. inexpectatus*. From a total of 704 individualised egg capsules collected in 2012, 29.1% and 12.3% were parasitised by *A. tasmaniae* and *A. inexpectatus*, respectively. Another 2.5% egg capsules originated *A. nitens*, *Centrodora damoni* (Girault) (Hymenoptera: Aphelinidae) (Ward et al. 2016), *Cirrospilus* sp. (Hymenoptera: Eulophidae), *Euderus* sp. (Hymenoptera: Eulophidae), and an unidentified Tetrastichinae (possibly a gall

inducer). Three larval parasitoid species were recovered, namely *Entedon magnificus* (Girault and Dodd) (Hymenoptera: Eulophidae), *Oxyserphus* sp. (Hymenoptera: Proctotrupidae), and *Anagonia* sp. (Diptera: Tachinidae). Based on 868 *Gonipterus* spp. larvae collected in 2012, the most abundant larval parasitoid was *E. magnificus* (9.2% of the larvae), whereas *Oxyserphus* sp. and *Anagonia* sp. parasitised 1.8% and 1.5% of the larvae, respectively. *Anaphes tasmaniae*, *A. inexpectatus*, *C. damoni*, and *Cirrospilus* sp. accepted and developed in *G. platensis* eggs, but only *A. inexpectatus* provided stable laboratory populations, which have been reared continuously since 2010. Seven laboratory populations of *A. inexpectatus* were established (Table 3.1) to preserve the species genetic pool.

3.2. Thermal requirements

Immature development

In this trial, complete development was not recorded for *A. inexpectatus* or *A. nitens* at 5 °C. Because stages prior to the pupa were not recorded, it is unclear whether larval eclosion and development occurred. In the 10-25 °C range, development times were longer for *A. nitens* than for *A. inexpectatus* (Table 3.2, $F_{1,4528} = 1452.6$, $p < 0.01$), and in both species they were longer for females than for males ($F_{1,4528} = 46.2$, $p < 0.01$). At 30 °C, only a few *A. inexpectatus* emerged, and while a trend of longer development times in males was found (Table 3.2), it was not statistically significant ($F_{1,28} = 3.0$, $p = 0.09$). LDT were estimated at 5.4 and 6.0 °C for *A. nitens* and *A. inexpectatus*, respectively (Fig. 3.1), and SET were estimated at 263 DD for *A. inexpectatus* and 313 DD for *A. nitens*.

Table 3.2 Development time (mean \pm SE) from egg to adult for *Anaphes inexpectatus* and *A. nitens* reared on *Gonipterus platensis* eggs, at six temperatures.

Species	Temperature (°C)	Number of emerging insects (females/males)	Development time (days)	
			Females	Males
<i>A. inexpectatus</i>	5		No development detected	
	10	562 (332/230)	61.6 \pm 0.25 a	60.7 \pm 0.28 b
	15	362 (213/149)	31.8 \pm 0.15 a	31.0 \pm 0.15 b
	20	269 (184/85)	18.5 \pm 0.14 a	18.1 \pm 0.20 a
	25	225 (149/76)	14.1 \pm 0.11 a	13.5 \pm 0.17 b
	30	30 (25/5)	12.9 \pm 0.41 a	15.0 \pm 1.90 a
<i>A. nitens</i>	5		No development detected	
	10	1,060 (772/288)	70.8 \pm 0.14 a	70.0 \pm 0.22 b
	15	500 (352/148)	33.7 \pm 0.14 a	32.7 \pm 0.13 b
	20	1,214 (873/341)	21.7 \pm 0.07 a	21.0 \pm 0.10 b
	25	337 (254/83)	16.5 \pm 0.11 a	15.8 \pm 0.18 b
	30		No adults emerged	

Different letters indicate significant differences in the duration of development of females and males, for each species and temperature combination (ANOVA, $p < 0.05$, $n = 100$ females).

Between 10 and 20 °C, most immatures (above 90%) of both species developed completely (Table 3.3). The proportion of adults that emerged at these temperatures ranged between 62% (15 and 20 °C) and 63% (10 °C) for *A. inexpectatus*, and between 39% (15 °C) and 83% (20 °C) for *A. nitens*. The proportion of *A. nitens* progeny that emerged at 15 °C was unexpectedly low. Above 20 °C, a steep decline in the percentage of fully formed and emerging progeny occurred in both species. At 30 °C, no *A. nitens* adults emerged and only 7% *A. inexpectatus* adults were able to emerge. The proportion of *G. platensis* unviable eggs was lower in non-parasitising females, particularly in *A. nitens*, as observed for example at 30 °C. In this situation, 23% and 6% unviable eggs were found in parasitising and non-parasitising females, respectively (Table 3.3), thus suggesting that up to 17% of the eggs may have been killed by females but no progeny developed.

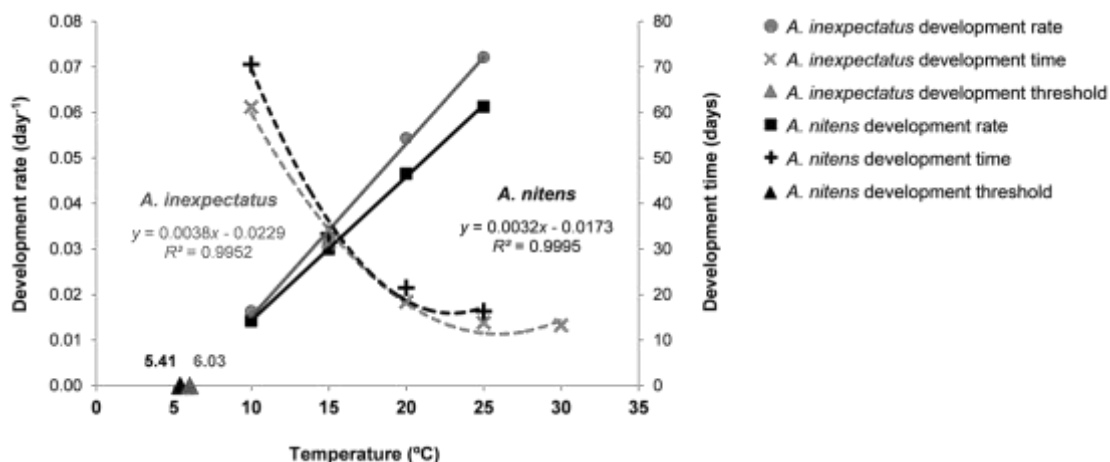


Fig. 3.1 Development rates, development times and estimated lower development thresholds from egg to adult for *Anaphes inexpectatus* and *A. nitens* reared on *Gonipterus platensis* eggs.

Adult performance and behaviour

Overall fecundity of *A. inexpectatus* (13.0 ± 0.99) was lower than that of *A. nitens* (21.7 ± 1.00) ($W_{1,498} = 42.7$, $p < 0.01$), and it was affected by temperature ($W_{5,498} = 236.5$, $p < 0.01$). Interaction between temperature and species was also significant ($W_{5,498} = 34.7$, $p < 0.01$). The highest fecundity was observed at 10 °C for *A. inexpectatus*, and at 20 °C for *A. nitens* (Table 3.4). The maximum number of eggs laid by a single female was 72 for *A. inexpectatus* (10 °C), and 70 for *A. nitens* (20 °C). Some females did not appear to have parasitised host eggs, particularly at 5 and 30 °C. At low and high temperatures (5, 10, and 25 °C) female fecundity of both *Anaphes* species displayed moderate to strong correlations with age (ranging from $R^2 = 0.43$ to $R^2 = 0.97$) (Fig. 3.2). At intermediate temperatures (15 and 20 °C) *A. inexpectatus* retained this egg laying pattern ($R^2 = 0.63$ at 15 °C and $R^2 = 0.96$ at 20 °C), while *A. nitens* fecundity was not correlated with female age ($R^2 \leq 0.03$) at 15 and 20 °C.

The number of parasitised eggs was lower for *A. inexpectatus* (7.9 ± 0.53) than for *A. nitens* (21.7 ± 1.06) ($W_{1,498} = 125.7$, $p < 0.01$), partly because the former species laid several eggs in the same host. Up to six emergences per host were recorded for *A. inexpectatus*, with an overall average of 1.6 ± 0.03 . Temperature ($W_{5,498} = 201.7$, $p < 0.01$) and the interaction between temperature and species ($W_{5,498} = 30.1$, $p < 0.01$)

were also significant (Table 3.4). Differences between *Anaphes* species were less pronounced, although still significant, when considering successfully emerging progeny. The highest numbers of emerging progeny per female were obtained at 10 °C for *A. inexpectatus* and at 20 °C for *A. nitens* (Table 3.4).

Table 3.3 Effect of five rearing temperatures on the proportion of fully developed progeny (emerged or not), emerging progeny, and unviable eggs (for parasitising and non-parasitising females), for *Anaphes inexpectatus* and *A. nitens* reared on *Gonipterus platensis* eggs.

Species	Temperature (°C)	Fully developed progeny (%) ¹	Emerging progeny (%) ¹	Unviable <i>G. platensis</i> eggs (%) ²	
				Parasitising females	Non-parasitising females
<i>A. inexpectatus</i>	10	96 ± 0.6 a	63 ± 1.6 a	11 ± 0.7 a	7 ± 1.3 a
	15	93 ± 1.0 b	62 ± 2.0 a	13 ± 0.8 b	12 ± 1.3 b
	20	95 ± 1.0 a	62 ± 2.3 a	14 ± 0.8 b	8 ± 0.9 a
	25	89 ± 1.3 c	44 ± 2.1 b	20 ± 1.0 c	10 ± 1.0 ab
	30	82 ± 1.7 d	7 ± 1.1 c	19 ± 1.0 c	11 ± 0.9 b
<i>A. nitens</i>	10	94 ± 0.6 b	75 ± 1.2 b	10 ± 0.6 a	6 ± 1.1 a
	15	97 ± 0.5 a	39 ± 1.4 c	18 ± 0.8 b	7 ± 1.0 a
	20	95 ± 0.6 b	83 ± 1.0 a	16 ± 0.8 b	7 ± 2.2 a
	25	87 ± 1.1 c	39 ± 1.7 c	23 ± 0.9 c	13 ± 1.8 b
	30	43 ± 1.3 d	0	23 ± 0.9 c	6 ± 2.6 a

Different letters indicate significant differences between temperatures within each species (Generalised Linear Models with Binomial distribution, post-hoc LSD tests, $p < 0.05$, $n = 100$ females).

¹ Percentage of the number of parasitoids detected.

² Percentage of the total number of eggs dissected. Refers to the proportion of *G. platensis* eggs that failed to produce larvae and did not display evidence of having been parasitised. Data on non-parasitising females provides an estimate of naturally unviable host eggs while data on parasitising females includes hosts killed as a result of parasitoid activity.

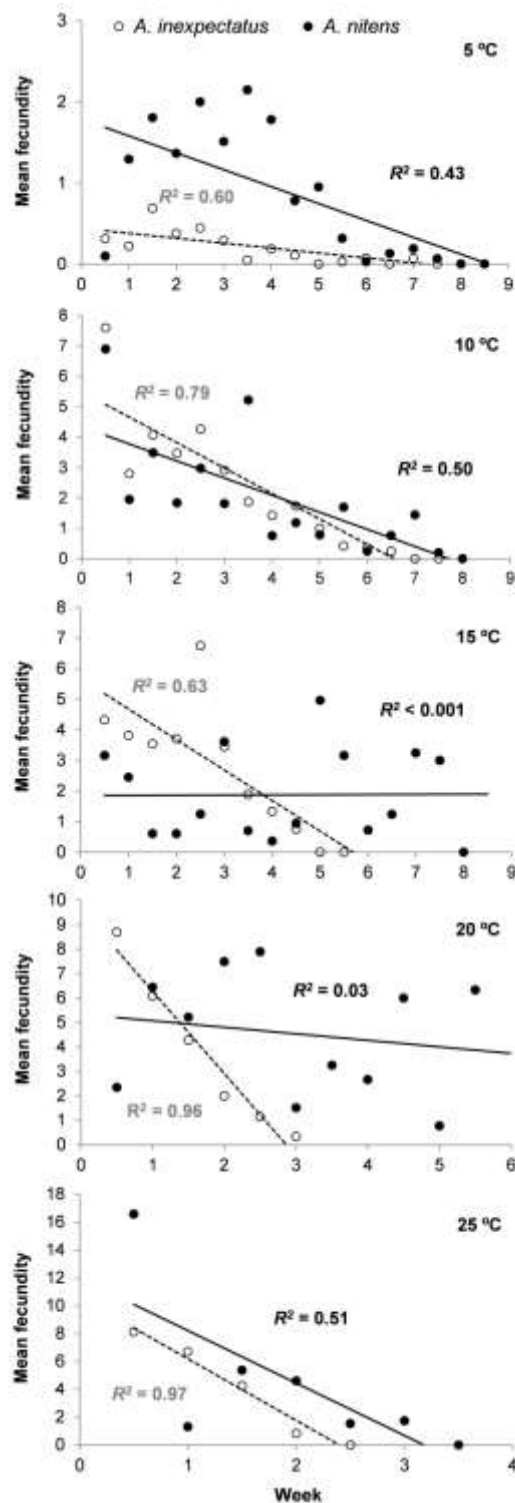


Fig. 3.2 Mean fecundity during female life, at five temperatures, of *Anaphes inexpectatus* and *A. nitens* reared on *Gonipterus platensis* eggs. Data at 30 °C is not shown due to short female longevity. Values of R^2 in black and solid lines refer to *A. nitens*; values of R^2 in grey and dashed lines refer to *A. inexpectatus*.

Table 3.4 Effect of six parasitism temperatures on fecundity (number of offspring detected), number of parasitised eggs, number of emerging adult progeny, percentage of fully developed progeny, and sex-ratio (mean \pm SE), for adults of *Anaphes inexpectatus* and *A. nitens* reared on *Gonipterus platensis* eggs.

Species	Temperature (°C)	Egg capsules offered ¹	Fecundity	Parasitised eggs	Emerging progeny	Fully developed progeny (%)	Sex-ratio (% of females)
<i>A. inexpectatus</i>	5	1,584	2.7 \pm 0.47 c	2.0 \pm 0.21 c	1.7 \pm 0.19 c	58 \pm 5.2 ab	62 \pm 4.7 a
	10	1,161	28.1 \pm 4.41 a	17.1 \pm 0.64 a	17.4 \pm 0.64 a	60 \pm 1.8 a	54 \pm 1.6 a
	15	486	14.7 \pm 2.39 b	8.9 \pm 0.47 b	8.3 \pm 0.45 b	60 \pm 2.6 a	56 \pm 2.2 a
	20	318	15.4 \pm 2.55 b	8.5 \pm 0.47 b	7.9 \pm 0.45 b	51 \pm 2.7 b	58 \pm 2.2 a
	25	318	15.8 \pm 2.57 b	9.6 \pm 0.49 b	7.3 \pm 0.43 b	51 \pm 2.6 b	58 \pm 2.2 a
	30	171	3.2 \pm 0.55 c	2.3 \pm 0.23 c	1.4 \pm 0.18 c	44 \pm 5.0 b	60 \pm 4.7 a
<i>A. nitens</i>	5	1,668	14.3 \pm 2.31 c	14.3 \pm 2.31 c	6.1 \pm 0.39 d	43 \pm 2.0 b	67 \pm 2.1 ab
	10	1,209	24.7 \pm 3.89 ab	24.7 \pm 3.89 ab	11.1 \pm 0.51 b	45 \pm 1.5 b	66 \pm 1.6 b
	15	1,368	20.7 \pm 3.27 bc	20.7 \pm 3.27 bc	9.6 \pm 0.48 c	47 \pm 1.7 b	71 \pm 1.6 a
	20	972	37.2 \pm 5.88 a	37.2 \pm 5.88 a	19.7 \pm 0.69 a	53 \pm 1.3 a	58 \pm 1.4 c
	25	618	29.3 \pm 4.72 a	29.3 \pm 4.72 a	12.5 \pm 0.56 b	42 \pm 1.4 b	65 \pm 1.4 b
	30	345	4.4 \pm 0.75 d	4.4 \pm 0.75 d	0.9 \pm 0.14 e	19 \pm 2.9 c	61 \pm 4.7 bc

Different letters indicate significant differences between temperatures for each species (Generalised Linear Models, with Negative Binomial distribution, post-hoc LSD tests, $p < 0.05$, $n = 40$ females)

¹ The number of egg capsules offered depended on female longevity, which decreased with temperature

The proportion of parasitised eggs leading to fully formed progeny was higher for *A. inexpectatus* than for *A. nitens* ($W_{1,820} = 125.7$, $p < 0.01$), and affected by temperature during oviposition ($W_{5,820} = 201.7$, $p < 0.01$) (Table 3.4). The interaction between temperature and species was also significant ($W_{5,820} = 30.1$, $p < 0.01$). In *A. inexpectatus*, egg viability declined linearly with increasing temperature, while in *A. nitens* egg viability displayed a unimodal pattern, with the maximum at 20 °C and a steep decrease at 30 °C (Table 3.4).

Progeny sex-ratio (percentage of females) was lower for *A. inexpectatus* (57.0% \pm 1.9) than for *A. nitens* (64.8% \pm 1.6) ($W_{1,401} = 17.9$, $p < 0.01$), but only marginally influenced by temperature ($W_{5,401} = 11.1$, $p = 0.05$). However, the interaction between temperature and species was significant ($W_{5,401} = 20.7$, $p < 0.01$). Whereas temperature had no effect on *A. inexpectatus* sex-ratio ($W_{5,186} = 5.7$, $p = 0.34$), a

significant effect was found for *A. nitens* ($W_{5,215} = 37.7$, $p < 0.01$), with the highest proportion of females at 15 °C (Table 3.4).

Net reproductive rates and intrinsic rates of natural increase were highest for *A. inexpectatus* at 10 and 15 °C and for *A. nitens* at 20 and 25 °C (Table 3.5). Generation times were shorter for *A. inexpectatus* at all temperatures. In both species, growth rates were close to zero at 5 °C and negative at 30 °C.

Temperature, species, gender, and feeding regime affected adult longevity (Fig. 3.3), with values decreasing as temperature increased for females ($b = -1.549 \pm 0.046$) and males ($b = -0.983 \pm 0.032$). Overall, *A. nitens* lived longer than *A. inexpectatus* for both females ($F_{1,1541} = 95.9$, $p < 0.01$) and males ($F_{1,1546} = 131.5$, $p < 0.01$). Females of both species lived longer than males ($F_{1,3031} = 46.4$, $p < 0.01$).

Table 3.5 Life table Jackknife estimates of population parameters (mean \pm SE) for *Anaphes inexpectatus* and *A. nitens*, reared on *Gonipterus platensis* eggs, at four temperatures.

Parameter	Temperature (°C)	<i>A. inexpectatus</i>	<i>A. nitens</i>	Significance level
Net reproductive rate (R_0) (number of females)	10	9.10 \pm 0.036	6.88 \pm 0.033	**
	15	4.15 \pm 0.019	3.78 \pm 0.183	*
	20	3.85 \pm 0.019	9.78 \pm 0.073	**
	25	3.65 \pm 0.023	7.01 \pm 0.065	**
Generation time (T) (days)	10	72.89 \pm 0.031	86.37 \pm 0.054	**
	15	40.20 \pm 0.024	48.43 \pm 0.730	**
	20	22.22 \pm 0.015	33.50 \pm 0.114	**
	25	18.39 \pm 0.010	21.85 \pm 0.038	**
Intrinsic rate of natural increase (r) (female.female ⁻¹ .day ⁻¹)	10	0.030 \pm 0.0001	0.022 \pm 0.0001	**
	15	0.035 \pm 0.0001	0.027 \pm 0.0004	**
	20	0.061 \pm 0.0002	0.068 \pm 0.0001	**
	25	0.070 \pm 0.0004	0.089 \pm 0.0004	**

Significant differences between species (Student t-test) are indicated as ** for $p < 0.01$ and * for $p < 0.05$, $n = 40$ females.

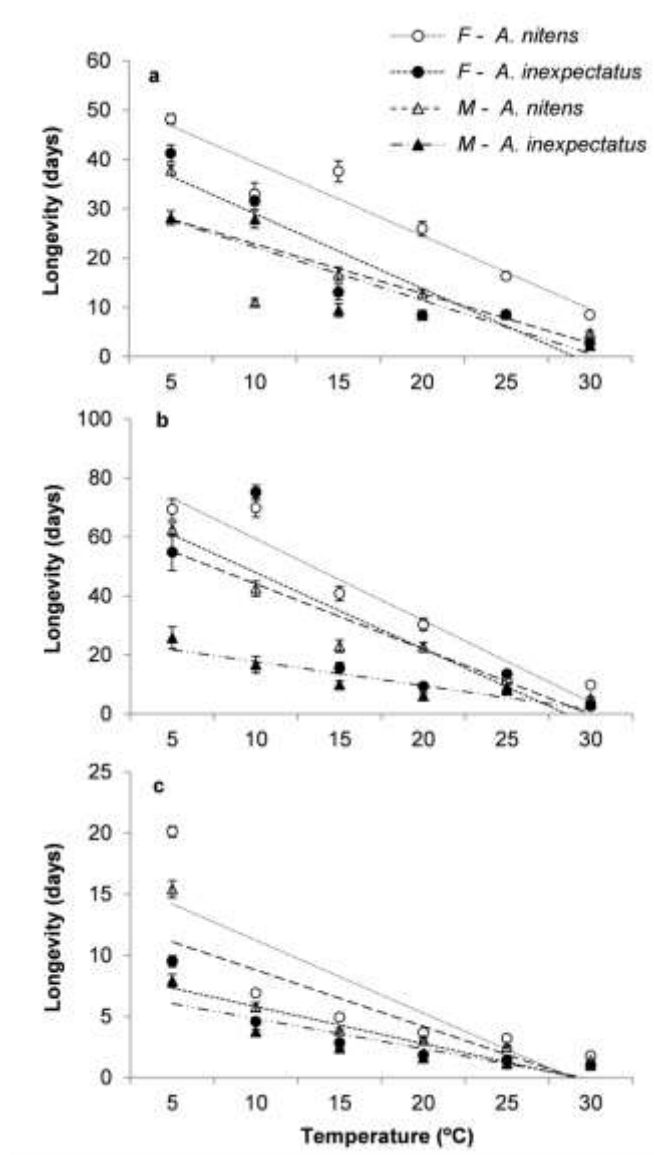


Fig. 3.3 Longevity (mean \pm SE) of females (F) and males (M) of *Anaphes inexpectatus* and *A. nitens* adults, reared on *Gonipterus platensis* eggs, at six temperatures and three food regimes (a: with honey and hosts; b: with honey and without hosts; c: without honey or hosts).

Food regimes influenced longevity for both females ($F_{2,1541} = 430.4$, $p < 0.01$) and males ($F_{2,1546} = 302.6$, $p < 0.01$). All parasitoids lived longer with honey in the absence of host eggs, and the shortest longevity was obtained when food was not provided. Females had the longest life spans at 5 °C, with food and without hosts (120 and 118 days for *A. inexpectatus* and *A. nitens*, respectively).

Searching behaviour frequencies were higher for *A. inexpectatus* than for *A. nitens* females at most temperatures (Table 3.6). In both species, low activity was observed at 5 °C, while reproductive behaviour frequencies (mating, antennation, and oviposition) were close to or over 50% at temperatures from 10 to 25 °C. *Anaphes nitens* females exhibited higher frequencies of reproductive behaviour than *A. inexpectatus* at 15 °C, while at 5, 20, and 30 °C *A. inexpectatus* had more reproductive contacts with hosts (Table 3.6).

Table 3.6 Proportion (mean \pm SE) of observations of searching and reproductive behaviour at six constant temperatures, for *Anaphes inexpectatus* and *A. nitens* reared on *Gonipterus platensis* eggs, based on 20 observations per female during the course of one hour.

Behaviour	Temperature (°C)	<i>A. inexpectatus</i>	<i>A. nitens</i>	Significance level
Searching	5	0.02 \pm 0.005	0.01 \pm 0.004	<i>ns</i>
	10	0.06 \pm 0.008	0.02 \pm 0.004	**
	15	0.26 \pm 0.015	0.08 \pm 0.010	**
	20	0.32 \pm 0.017	0.06 \pm 0.008	**
	25	0.19 \pm 0.014	0.14 \pm 0.012	*
	30	0.34 \pm 0.016	0.04 \pm 0.007	**
Reproduction	5	0.20 \pm 0.013	0.09 \pm 0.009	**
	10	0.46 \pm 0.017	0.47 \pm 0.017	<i>ns</i>
	15	0.46 \pm 0.017	0.69 \pm 0.016	**
	20	0.54 \pm 0.018	0.48 \pm 0.017	*
	25	0.66 \pm 0.017	0.69 \pm 0.016	<i>ns</i>
	30	0.19 \pm 0.013	0.13 \pm 0.012	**

Significant differences between species (Wald Chi-square) are indicated as ** for $p < 0.01$, * for $p < 0.05$, and *ns* for no significance, $n = 40$ females.

4. Discussion

Anaphes tasmaniae was the most abundant parasitoid of *Gonipterus* spp. in Tasmania. Efforts to establish a stable laboratory population were unsuccessful, even though *G. platensis* was accepted and suitable for development. In Chile, *A. tasmaniae* has been successfully reared and released, albeit in small numbers

(Mayorga et al. 2013). As for the second most common parasitoid, *A. inexpectatus*, laboratory populations were established easily, indicating it is well adapted to *G. platensis*. Although other egg parasitoids were found to successfully parasitise *G. platensis*, they collectively accounted for less than 2.5% egg parasitism in their native distribution and were considered to have less potential as biological control agents.

Parasitoid surveys reported here are consistent with previous findings. In southwestern Australia, apart from *A. nitens*, two egg parasitoids, *Euderus* sp. and *Centrodora* sp., have been reported (Loch, 2008). With regard to larval parasitoids, surveys in southeast Australia reported a tachinid fly (Tooke 1955). Three larval parasitoids were found in Tasmania, namely *Oxyserphus turneri* (Dodd) (Hymenoptera: Proctotrupidae), *Apanteles* sp. (Hymenoptera: Braconidae), and a tachinid (Tribe 2003), while in southwestern Australia an unidentified tachinid was reared from *G. platensis* (Loch 2008). In 2011, another larval parasitoid, *E. magnificus*, was collected in Tasmania from *Gonipterus* spp., and shown to successfully parasitise *G. platensis* (Gumovsky et al. 2015). We were however unable to confirm parasitism of *G. platensis* by larval parasitoids.

Temperatures ranging from 10 to 20 °C were found to be adequate for immature development in both *A. inexpectatus* and *A. nitens*. Results for *A. nitens* incubation at 15 °C were inconsistent, with only 39% of the parasitoids managing to emerge even though 97% of the progeny was fully formed. Temperature and relative humidity records in the climate chamber were confirmed to have been stable, and a new subset of insects was used to repeat the trial, with similar results. Working with the related species *Anaphes flavipes* (Forster), Anderson and Paschke (1969) suggested facultative diapause in this species can be induced at 15.5 °C. However, in a study on *A. nitens* ecology in northwestern Spain, Santolamazza-Carbone et al. (2009) found no evidence of diapause and suggest quiescence or oligopause as the overwintering strategy in this species. Further studies would be required to investigate the causes for the results at this temperature. When reared at 5 °C, immatures of both species failed to develop, as this temperature is below their estimated LDT. Even though *A. nitens* has been reported to tolerate short periods of exposure to temperatures below 0 °C (Tooke 1955; Santolamazza-Carbone et al. 2009), prolonged incubation at 5 °C proved lethal. At 25 °C our results indicate

deleterious effects in both species, as shown by the decrease in the proportion of fully formed and emerging progeny (Table 3.3). At 30 °C, deleterious effects of temperature were clear, particularly in *A. nitens*, where only 43% of the immatures fully developed and no adult emergence was recorded. In *A. inexpectatus*, 82% of the immatures were fully developed and 7% of the adults emerged, indicating that it is somewhat more tolerant to high temperature.

In poikilothermic organisms, LDT tends to decrease and SET to increase with increasing latitude, thus reflecting adaptation to the local thermal environment (Honek 1996; Trudgill et al. 2005). Several studies have focused on the thermal biology of *Anaphes* species (Anderson and Paschke 1969; Stoner and Surber 1969; Leibee et al. 1979; Collins and Grafius 1986; Jackson 1987; Santolamazza-Carbone et al. 2006; Traoré et al. 2006). LDT was found to range from 5.4 °C in an *Anaphes listronoti* Huber strain from northern USA (Collins and Grafius 1986) to 10.1 °C in an *Anaphes victus* Huber strain from southern USA (Traoré et al. 2006). Development thresholds estimated for *A. inexpectatus* and *A. nitens* in the present study (6.0 and 5.4 °C, respectively) are close to the minimum range reported for *Anaphes* spp., suggesting adaptation to cool environments in both species. Because LDT values cannot be determined precisely (Campbell et al. 1974; Lamb 1992), and because the difference between *Anaphes* species was marginal, it is unclear whether field performance at low temperature will be affected. Among other *Anaphes* spp., SET has been found to vary between 131 DD in *A. victus* (Traoré et al. 2006) and 270 DD in *Anaphes diana* (Girault) (Leibee et al. 1979). While for *A. inexpectatus* SET was estimated within this range (263 DD), in *A. nitens* the estimated 313 DD resulted in longer generation times. On average, *A. inexpectatus* generation times were roughly 20% shorter than those of *A. nitens*. A similar value of 318 DD had been reported for *A. nitens* (Santolamazza-Carbone et al. 2006). Males emerged before females, as reported for other *Anaphes* species (Tooke 1955; Anderson and Paschke 1969; Jackson 1987; Traoré et al. 2006), which is a common feature among gregarious and quasi-gregarious parasitoids whenever mating between siblings occurs upon emergence (Hamilton 1967; Hardy et al. 2005). Development times for the various stages of *A. inexpectatus* development would be interesting to investigate in future studies.

Average female fecundity ranged between 14.7 ± 2.39 and 28.1 ± 4.41 offspring per female in *A. inexpectatus* and between 20.7 ± 3.27 and 37.2 ± 5.88 in *A. nitens* (excluding extreme temperatures of 5 and 30 °C). These ranges are consistent with previous findings in *A. nitens* (Tooke 1955; Santolamazza-Carbone and Cordero-Rivera 2003a) and other *Anaphes* species (Stoner and Surber 1969; Ahmad 1978; Aeschlimann et al. 1989). *Anaphes nitens* females parasitised more eggs than *A. inexpectatus* at all temperatures, which is not surprising, as gregarious parasitoids are often smaller, less fecund, and shorter lived than solitary ones (Collins and Grafius 1986; Boivin and Baaren 2000). However, *A. inexpectatus* produced more progeny at 10 °C and displayed a higher proportion of fully formed progeny, particularly at higher temperatures. Our results indicate that short periods of exposure to high temperature (up to four days at 30 °C) resulted in significantly decreased immature viability in *A. nitens*.

The majority of studies with *Anaphes* have found most parasitism to occur within the first 24-72 h after emergence (Collins and Grafius 1986; Jones and Jackson 1990). In the present study, *A. inexpectatus* and *A. nitens* displayed decreasing fecundity with increasing age at most temperatures. At 5 and 10 °C, female longevity was prolonged and fewer eggs were laid towards the last weeks of life. At 25 °C egg fecundity was apparently unaffected by the shorter lifespan. However, at moderate temperatures of 15 and 20 °C, *A. nitens* females continued to lay eggs throughout their life, unlike *A. inexpectatus* (Fig. 3.2). Santolamazza-Carbone and Cordero-Rivera (2003a) concluded that *A. nitens* is weakly synovigenic (i.e. can mature eggs during its lifetime) in a study performed at 21 °C. The fact that *A. nitens* fecundity was unaffected by female age only at moderate temperatures suggests this ability depends on temperature, but our data does not provide clues to a possible explanation. Although ovarian eggs were not counted, the egg laying pattern displayed by *A. inexpectatus* females suggests that this species is mostly proovigenic (i.e. ovigenesis is complete prior to or shortly after emergence and females don't mature additional eggs). Ovarian dissection studies would be needed to clarify this trait.

Because multiple *A. inexpectatus* immatures can develop within a single host egg (up to six in the present study), a female's fecundity will exceed the number of parasitised eggs, which has implications on pest control. A single *A. nitens* can

parasitise more eggs and cause higher pest mortality when females are not host limited. Inversely, when hosts are scarce, *A. inexpectatus* females can maximise their fecundity per host, thus enhancing their chances of persisting.

Fecundity and the number of parasitised eggs were probably underestimated, as several host eggs were likely parasitised but immatures were not detected. Such mortality probably resulted from low vitality, lethal effects of temperature, or superparasitism; the later known to occur in *A. nitens* (Tooke 1955; Hanks et al. 2000; Santolamazza-Carbone and Cordero-Rivera 2003a). Although deleterious effects of high temperature seemed more pronounced in *A. nitens* than in *A. inexpectatus*, superparasitism may have further contributed to differences in egg viability between species. Negative effects of superparasitism are less likely in *A. inexpectatus*, as this species is gregarious and multiple parasitoids can develop successfully in a single host.

In both species, females lived longer than males, which is consistent with previous studies on *Anaphes* spp. (Ahmad 1978; Jackson 1987; Santolamazza-Carbone et al. 2009). Adult feeding increased longevity at all temperatures. On average, life expectancy of unfed adults was about 20% of that of fed parasitoids. Parasitoids commonly use a variety of sugar based non-host foods, which increase their reproductive ability by prolonging longevity and providing more time to find hosts (Jones and Jackson 1990; Williams and Roane 2007; Wade et al. 2008; Lundgren 2009). Under field conditions, nectar is one of the most common food sources, and *A. nitens* is likely to feed on eucalypt flowers (Tooke 1955; Santolamazza-Carbone et al. 2009). Additional food sources may be provided by honeydew (Williams and Roane 2007; Lundgren 2009), which is common in eucalypt stands as a result of psyllid infestation. Because honeydew's quality as a food source is frequently lower than that of nectar (Williams and Roane 2007; Lundgren 2009), additional studies are necessary to determine its effect on *Anaphes* performance. Longevity was highest when parasitoids were provided with honey, but no hosts. For females, it's been argued this derives from not expending energy in oviposition (Sahad 1984; Santolamazza-Carbone et al. 2009). However, our results indicate males also lived longer in the absence of host eggs, and it is likely that disturbance during handling for host egg replacement reduced vitality.

Partly because *A. inexpectatus* had shorter development times, intrinsic rates of natural increase were higher for this species at lower temperatures (10 and 15 °C), and higher for *A. nitens* at higher temperatures (20 and 25 °C). When wild specimens were introduced into the laboratory, temperatures for each species were adjusted to obtain maximum fecundity (10 °C for *A. inexpectatus* and 20 °C for *A. nitens*). It is therefore possible that acclimatisation to rearing temperatures for consecutive generations may have amplified differences between species (Laudien 1973; Bloem and Yeargan 1982).

Anaphes nitens had a lower LDT, predictably initiating its development before *A. inexpectatus* in late winter, at the beginning of *G. platensis* egg laying. In addition, *A. nitens* displayed higher fecundity than *A. inexpectatus* at 5 °C, which further suggests that the former species is well adapted to low temperature. *Anaphes inexpectatus* developed faster and displayed maximum net reproduction rate at the critical temperature of 10 °C, while at moderate temperature regimes (20-25 °C) *A. nitens* was generally more efficient. These results suggest that *A. inexpectatus* may complement the biological control of *G. platensis* already exerted by *A. nitens* early in the growing season, while *A. nitens* will likely outcompete the former species during spring. Besides data on thermal biology of both species, competition studies are required to provide insight on the outcome of the interaction between *A. inexpectatus* and *A. nitens*. Several aspects suggest that *A. inexpectatus* is likely to establish and disperse in eucalypt plantations in Portugal, namely its tolerance to high temperature (30° C), while being active and able to parasitise at low temperature (5 °C), but ultimately this can only be assessed through field release studies.

Additional natural enemies should not be discarded if stable biological control of *G. platensis* is to be achieved over its entire distribution range. In particular, larval parasitoids are interesting because they attack a development stage that is currently free of natural enemies outside Australia.

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CHAPTER 4

Assessing the competitive interactions between two egg parasitoids of the *Eucalyptus* snout beetle, *Gonipterus platensis*, and their implications for biological control

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Assessing the competitive interactions between two egg parasitoids of the *Eucalyptus* snout beetle, *Gonipterus platensis*, and their implications for biological control

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Abstract

The *Eucalyptus* snout beetle, *Gonipterus platensis* (Marelli), is an important pest of *Eucalyptus globulus* Labill.. This insect is partially controlled by the egg parasitoid *Anaphes nitens* (Girault) in many regions, but the introduction of additional natural enemies can potentially increase pest control. In this study, we evaluate intra- and interspecific competitive interactions between the incumbent *A. nitens* and the new egg parasitoid *Anaphes inexpectatus* Huber and Prinsloo. The effects of temperature, order of parasitism, number of parasitoid ovipositions, time interval between ovipositions, and host egg age were analysed. Distinct outcomes of competition were found at different temperatures, with benefit to *A. inexpectatus* at 20 °C. The first species to parasitise generally prevailed over the second, indicating exploitation competition. However, interference competition was also apparent, namely when *A. inexpectatus* laid multiple eggs, outcompeting *A. nitens*, and when the first parasitism occurred six days before. In this case, the second species was able to eliminate the first. *Anaphes nitens* tended to reject eggs parasitised by *A. inexpectatus*, whereas *A. inexpectatus* showed no interspecific host discrimination behaviour towards eggs parasitised by *A. nitens*. Overall, *A. nitens* parasitised more hosts and is expected to contribute more to pest control, but it was found to be more susceptible to intraspecific competition. Results suggest that *A. inexpectatus* and *A. nitens* should be able to coexist, as asymmetric competition was found to depend on temperatures. However, *A. inexpectatus* establishment in the field in areas where *A. nitens* is already present may be delayed or even prevented due to interspecific

competition. As such, the introduction of *A. inexpectatus* in a classical biological control programme against *G. platensis* is advised to be carried out by releasing large numbers of parasitoids in consecutive occasions.

Keywords

Curculionidae, Mymaridae, Anaphes, classical biological control, competition.

1. Introduction

Classical biological control (CBC) aims to reduce the population density of pests through the introduction of natural enemies, to a level at which they no longer cause economic damage (Waage and Mills 1992). Although a single natural enemy may adequately suppress an invasive pest, multi-species introductions are a frequent practice in CBC (Waage and Mills 1992). However, the introduction of additional agents does not necessarily enhance control, as many fail to establish (Ehler and Hall 1982; Denoth et al. 2002) and antagonistic interactions between the released natural enemies may occur (Pedersen and Mills 2004). A common antagonistic effect between introduced agents is competition, which in some cases may lead to competitive displacement or even exclusion of previously introduced agents or native natural enemies (DeBach 1966; Ehler and Hall 1982; Mills 2006). Some introduced agents may even have an adverse impact on overall pest control and a few examples are known from previous CBC programmes, including the introduction of hyperparasitoids (Nguyen et al. 1983) and kleptoparasitoids (Schröder 1974). Despite the potential existence of antagonistic interactions, the overall effect of natural enemy coexistence in the context of biological control is generally considered beneficial or inconsequential, when measured in terms of pest population regulation (DeBach 1966; Pedersen and Mills 2004; Mills 2006). The introduction of additional natural enemies leads, more often than not, to increased pest mortality (Stiling and Cornelissen 2005). Nevertheless, the introduction of multiple agents should be conducted with caution and the evaluation of potential interactions should be addressed (Denoth et al. 2002; Pedersen and Mills 2004; Ardeh et al. 2005; Mills 2006).

Insect parasitoids can compete directly for host resources, influencing the dynamics of plant-herbivore-parasitoid systems (Godfray 1994; De Moraes et al. 1999). Competitive interactions reduce the reproductive success of each parasitoid species and consequently affect pest control (De Moraes et al. 1999; Boivin and Brodeur 2006; Cusumano et al. 2012; Harvey et al. 2013). Direct competition may take place among adults searching for hosts (extrinsic competition) or among parasitoid larvae developing on or inside the host (intrinsic competition) (De Moraes et al. 1999; Harvey et al. 2013). Additionally, insect parasitoids are particularly prone to experiencing indirect competition since their hosts remain available in the environment and can be exploited by competitors from the same species (intraspecific competition) or of different species (interspecific competition). When a female parasitoid enters a patch that has been previously parasitised, she may adjust her oviposition behaviour (indirect competition) (Boivin and Brodeur 2006). As a result, hosts may be attacked by multiple females of the same species (superparasitism) or of different species (multiparasitism) (Godfray 1994).

The *Eucalyptus* snout beetle, *Gonipterus platensis* (Marelli) (Coleoptera: Curculionidae), is a Tasmanian weevil that became a key pest of eucalypts outside its native range. This insect is widely distributed, occurring in Western Australia, New Zealand, Southwestern Europe (Portugal and Spain), North America (California and Hawaii), and South America (Argentina, Brazil, and Chile) (Mapondera et al. 2012). *Gonipterus platensis* populations have been partially or completely controlled in these regions using the CBC agent *Anaphes nitens* (Girault) (Hymenoptera: Mymaridae), imported from Australia (Valente et al. 2018). Despite the good results obtained with *A. nitens*, successful control has not been achieved everywhere, especially in some regions of South America (Gumovsky et al. 2015), Western Australia (Loch 2008), and Southwestern Europe (Cordero-Rivera et al. 1999, Reis et al. 2012, Valente et al. 2018). Different climatic requirements of *G. platensis* and *A. nitens* and asynchrony between oviposition by the snout beetle and the parasitoid may explain the insufficient efficacy of *A. nitens* in those regions (Tribe 2003, Loch 2008, Reis et al. 2012). In Southwestern Europe, Reis et al. (2012) found that parasitism in early spring decreased along an altitude gradient. Furthermore, average maximum temperatures during winter months below a threshold temperature of 10 °C resulted in low parasitism rates of *G. platensis* by *A. nitens* during late winter (10.1%),

while temperatures above 11.5 °C resulted in 70.9% parasitism. These results suggest that winter temperature plays an important role in the success of biological control of *G. platensis*.

Due to unsatisfactory success of *A. nitens*, other Australian natural enemies have been studied aiming to improve CBC of the snout beetle (Huber and Prinsloo 1990; Mayorga et al. 2013; Gumovsky et al. 2015; Valente et al. 2017b). One of these natural enemies is the egg parasitoid *Anaphes inexpectatus* Huber and Prinsloo (Hymenoptera: Mymaridae). While *A. nitens* is a solitary endoparasitoid (only one individual emerges per host), *A. inexpectatus* is gregarious, with up to six individuals being able to develop in a single host egg (Huber and Prinsloo 1990, Valente et al. 2017b). These two *Anaphes* species are allopatric. *Anaphes nitens* is native to Australian mainland while the native range of *A. inexpectatus* is Tasmania (Mapondera et al. 2012). Although *A. nitens* has been detected in Tasmania, it is likely a recent introduction from Australian mainland (Valente et al. 2017b). Laboratory studies suggest that *A. inexpectatus* is a promising biological control candidate against *G. platensis* in the colder regions of Southwestern Europe, partly because of its higher net reproductive rate than *A. nitens* at low temperatures (Valente et al. 2017a, 2017b). However, how the introduction of *A. inexpectatus* may affect biological control by the already established *A. nitens* is unknown.

In a CBC programme, it is crucial to consider potential antagonistic effects with already established biological control agents. Two main questions arise, namely: i) will the new natural enemy compete with the existing (incumbent) species?; and ii) will competition affect the interacting natural enemies and the suppression of the shared host pest? In order to provide insight into these questions, this study focused on the competitive interactions between two exotic parasitoids of the *Eucalyptus* snout beetle, the incumbent *A. nitens* and the new *A. inexpectatus*. A series of trials was performed to test the end result of indirect (between parasitising females) and direct (between developing larvae) intra- and interspecific competition in *A. nitens* and *A. inexpectatus*, on naturally laid egg capsules. Since parasitoid performance and ultimately the outcome of competition may depend on climate conditions, particularly temperature, trials were performed at 10 °C (critical winter temperature) and 20 °C (typical spring temperature). A second set of tests was conducted using individual host eggs (to determine which eggs were parasitised by which female

parasitoid) in order to evaluate intra- and interspecific host discrimination and larval competition. The results are discussed in light of the best available information on the biology of *A. nitens* and *A. inexpectatus*, and predictions are made on how the introduction of *A. inexpectatus* in areas where *A. nitens* is already established may affect parasitoid populations and biological control of *G. platensis*.

2. Material and Methods

2.1. Insect rearing

Anaphes inexpectatus adults were obtained from populations collected in Tasmania, Australia, between 2010 and 2016 (Valente et al. 2017b) and maintained at RAIZ (Research Institute for Forestry and Paper, Portugal) under quarantine laboratory conditions (10 °C, 60-80% RH, and 14:10 L:D photoperiod). *Anaphes nitens* adults were obtained from populations collected in Portugal (Barcelos) and maintained under laboratory conditions (20 °C, 60-80% RH, and 14:10 L:D photoperiod). These are the standard rearing conditions used at RAIZ laboratory rearing facility, as they maximise female fecundity for both species (Valente et al. 2017b). Newly emerged parasitoids were placed in glass vials (18 mm diameter, 180 mm long) together with *G. platensis* egg capsules (on average, each egg capsule contains eight to ten eggs) and a droplet of honey solution (50% in water). Parasitoids were allowed access to host eggs for up to 5 days, after which the egg capsules were incubated at 15 °C in plastic boxes until parasitoid emergence. The egg capsules used in rearing and trials were obtained from field collected *G. platensis* adults maintained in the laboratory (at the same environmental conditions as *A. nitens*), in 1 L perforated plastic boxes with 3-5 *Eucalyptus globulus* Labill. shoots.

2.2. Outcome of competition at two temperatures

In the first set of trials, one fresh (under 6-hour old) *G. platensis* egg capsule was offered to inexperienced and previously mated female parasitoids, following the scheme shown in Fig. 4.1. Parasitism in the absence of competition was assessed by allowing a single female of *A. inexpectatus* (In) or *A. nitens* (Ni) to parasitise for a 4-hour period. The outcome of competition was assessed through sequential parasitism (4-hour periods) for all combinations of parasitoid species (In/In, Ni/Ni,

In/Ni, and Ni/In) or simultaneous parasitism by one female of each species (Simultaneous) for a similar 4-hour period. All tests were performed in small glass tubes (12 mm diameter, 100 mm long). Parasitoids were provided with honey solution (50% in water) as food during the experiments. The trials were performed at two temperatures, 10 °C and 20 °C, in a climatic chamber (Fitoclima 1200S). One hundred trials per treatment and temperature were performed, but egg capsules that showed no evidence of parasitism were excluded resulting in 26 to 85 replicates. All egg capsules were incubated at the tested temperature (10 °C or 20 °C; 60-80% RH and 14:10 L:D photoperiod) until progeny emerged.

2.3. Factors affecting host discrimination and the outcome of competition

Because host egg capsules are opaque, it is impossible to determine which individual egg was parasitised by each female parasitoid. Therefore, in this set of trials, choice tests were performed using individual eggs removed from egg capsules. These were obtained by carefully dissecting freshly laid egg capsules in which the outer casing had not hardened. Under 48-hour old, mated, and inexperienced parasitoid females were offered one previously parasitised egg (PE) and one unparasitised egg (UE). PEs were obtained by allowing one mated female to parasitise an egg once (In1 and Ni, for *A. inexpectatus* and *A. nitens*, respectively) or multiple times for the gregarious *A. inexpectatus* only (In+). Females of *A. inexpectatus* were removed immediately after a first parasitism was confirmed in order to obtain eggs parasitised only once. To investigate the effect of delayed oviposition on host discrimination and suitability, UEs and PEs were incubated at 20 °C, 60-80% RH, and 14:10 L:D photoperiod for under six hours (<6h), one day (1d), three days (3d), or six days (6d) before the tests. Different combinations of species and number of ovipositions were tested, totalling eight treatments (Table 4.1).

Each female parasitoid was placed in the centre of a small glass petri dish (50 mm diameter, 15 mm height) with a white cardstock strip (5 mm wide, 50 mm long) containing one PE and one UE of the same age ca. 2 cm apart, attached with a droplet of diluted gum Arabic. Placement of strips and petri dishes was randomized in each replicate to avoid possible differences in shading, which could bias the results. Parasitoids were provided with honey solution (50% in water) as food for the duration of the experiments. Behaviour was recorded as soon as females were introduced in

the petri dishes and throughout the experiment. The following information was recorded: i) first egg selected for parasitism; ii) rejection after external inspection with the antennae (antennation); iii) rejection after internal inspection with the ovipositor (probing); and iv) oviposition, confirmed by abdominal contractions [for details on oviposition behaviour see Santolamazza-Carbone et al. (2004)]. Each test ended when the female parasitised both eggs or after a 4-hour period, when the female rejected one or both eggs. For each treatment, tests were replicated until at least 10 females had accepted both eggs, up to a maximum of 50 trials.

Additionally, the viability for parasitoid development of *G. platensis* eggs with different ages (<6h, 1d, 3d, and 6d) was tested. Individual eggs were attached with a droplet of diluted gum Arabic to a white cardstock strip (5 mm wide, 50 mm long) and offered to one mated and inexperienced female parasitoid (one strip with one egg per female). Parasitism was confirmed by direct observation. A total of 69 to 181 eggs were tested, per *Anaphes* species and host age.

All observations were performed at room temperature (ca. 20 °C, controlled by air conditioning). All eggs were incubated at 20 °C, 60-80% RH, and 14:10 L:D photoperiod until progeny emerged.

Type	Treatment	4 hours	4 hours
No competition	In	1 ♀ <i>A. inexpectatus</i>	
	Ni	1 ♀ <i>A. nitens</i>	
Intraspecific competition	In/In	1 ♀ <i>A. inexpectatus</i>	1 ♀ <i>A. inexpectatus</i>
	Ni/Ni	1 ♀ <i>A. nitens</i>	1 ♀ <i>A. nitens</i>
Interspecific competition	Ni/In	1 ♀ <i>A. nitens</i>	1 ♀ <i>A. inexpectatus</i>
	In/Ni	1 ♀ <i>A. inexpectatus</i>	1 ♀ <i>A. nitens</i>
	Simultaneous	1 ♀ <i>A. inexpectatus</i> + 1 ♀ <i>A. nitens</i>	

Fig. 4.1 Treatments used to evaluate the outcome of intra- and interspecific competition between *Anaphes inexpectatus* and *A. nitens* on *Gonipterus platensis* egg capsules.

Table 4.1 Treatments used to evaluate host discrimination and intra- and interspecific competition between *Anaphes inexpectatus* and *A. nitens* on individual eggs of *Gonipterus platensis*.

Type	Treatment	First parasitising female		Second parasitising female	
		Species	Number of ovipositions	Species	Number of ovipositions
Intraspecific competition	In1/In1	<i>A. inexpectatus</i>	1	<i>A. inexpectatus</i>	1
	In1/In+	<i>A. inexpectatus</i>	1	<i>A. inexpectatus</i>	2 or more
	In+/In1	<i>A. inexpectatus</i>	2 or more	<i>A. inexpectatus</i>	1
	Ni/Ni	<i>A. nitens</i>	1	<i>A. nitens</i>	1
Interspecific competition	Ni/In1	<i>A. nitens</i>	1	<i>A. inexpectatus</i>	1
	Ni/In+	<i>A. nitens</i>	1	<i>A. inexpectatus</i>	2 or more
	In1/Ni	<i>A. inexpectatus</i>	1	<i>A. nitens</i>	1
	In+/Ni	<i>A. inexpectatus</i>	2 or more	<i>A. nitens</i>	1

2.4. Data analysis

The number of *G. platensis* larvae and emerging parasitoid progeny was recorded in every experiment. Individual eggs and egg capsules were observed under a stereomicroscope (Leica MZ8) to determine parasitism status. Progeny per female was calculated as the sum of emerging progeny and fully developed adults that failed to emerge.

Outcome of competition at two temperatures: the proportion of eggs parasitised by *A. nitens* or *A. inexpectatus* in relation to the total number of eggs parasitised was analysed by General linear models (GLM) using Binomial probability distribution and log link function, considering the factor treatment. Differences in the number of eggs parasitised per female by each species were determined by paired samples Wilcoxon Signed Ranks tests, for each temperature and treatment. GLM were also used to determine the effect of temperature and parasitism order on fully developed progeny per female of each species. Models were first applied with two factors (temperature and parasitism treatment) and interaction term. Since the interaction term was significant, each temperature was analysed separately. Differences between treatments at each temperature were then analysed by pairwise comparison using Least Significant Difference (LSD) tests.

Factors affecting host discrimination and the outcome of competition: the proportion of eggs (PEs and UEs) rejected by each species for each parasitism combination and egg age was analysed with Wilcoxon Signed Rank test. The frequency at which each egg (PE and UE) was selected first for parasitism by each species was analysed with a Binomial distribution test, considering equal probability of choice (0.5). GLM with Binomial distribution was used to determine the effect of parasitism combination and the interval between ovipositions on the probability of a previously parasitised egg being accepted for parasitism. Because female behaviour towards host eggs was not affected by the number of times *A. inexpectatus* parasitised (once or multiple times), the results were grouped together in the analysis. Differences in the number of *A. inexpectatus* or *A. nitens* emerging per multiparasitised host egg were compared by χ^2 tests, for each parasitism order combination. In both *Anaphes* species, suitability for parasitism of eggs of different ages was analysed with GLM with Binomial distribution and post-hoc LSD tests. All analyses were performed with IBM SPSS Statistics 25 (SPSS, Chicago, IL, USA).

3. Results

3.1. Outcome of competition at two temperatures

The proportion of *G. platensis* eggs originating *A. nitens* or *A. inexpectatus* was affected by which species parasitised first, at both 10 °C and 20 °C ($W_2= 12.105$, $p= 0.002$ and $W_2= 173.423$, $p< 0.001$, respectively; Fig. 4.2). At 10°C, significant differences were found only when *A. nitens* parasitised first ($Z_{54, \text{two-sided}}= -3.883$, $p< 0.001$), resulting in 3.1 ± 0.4 eggs parasitised by *A. nitens* versus 0.9 ± 0.2 eggs parasitised by *A. inexpectatus*, on average. Differences were not significant when both species parasitised simultaneously ($Z_{58, \text{two-sided}}= -1.126$, $p= 0.260$) or when *A. nitens* parasitised after *A. inexpectatus* ($Z_{40, \text{two-sided}}= -1.555$, $p= 0.120$). At 20 °C, the outcome of parasitism was skewed towards the species that parasitised first (Fig. 4.2). When *A. nitens* parasitised first, 2.8 ± 0.3 eggs originated this species while 1.0 ± 0.2 originated *A. inexpectatus*, on average ($Z_{80, \text{two-sided}}= -5.568$, $p< 0.001$). Conversely, when *A. inexpectatus* parasitised first, 2.7 ± 0.3 eggs originated this species whereas 0.4 ± 0.1 originated *A. nitens*, on average ($Z_{83, \text{two-sided}}= -4.166$,

$p < 0.001$). When parasitism was simultaneous, 2.3 ± 0.3 eggs were parasitised by *A. inexpectatus* while 1.5 ± 0.3 eggs were parasitised by *A. nitens*, on average, resulting in significant differences between species ($Z_{64, \text{two-sided}} = -2.292$, $p = 0.022$). Overall, *A. inexpectatus* outcompeted *A. nitens* at 20°C (Fig. 4.2).

The results on the emerging progeny per *Anaphes* species followed a similar trend to those on the number of parasitised eggs at each temperature and parasitism treatment (Table 4.2). At 10 °C, in the absence of competition, each *A. nitens* female produced 4.19 ± 0.69 offspring on average. However, offspring production was significantly reduced by 53% under intraspecific competition (Table 4.2). Under interspecific competition, *A. nitens* progeny was reduced by 55% and 64%, when parasitising simultaneously and after *A. inexpectatus*, respectively. But when *A. nitens* was allowed parasitising first progeny production was only reduced by 26%. At 20 °C, all types of competition decreased the fitness of *A. nitens* females. Progeny per female in the absence of competition was 3.97 ± 0.56 , and it was reduced by 27% (intraspecific competition), and by 29%, 63%, and 89%, when parasitising before, simultaneously or after *A. inexpectatus* (interspecific competition), respectively.

Intraspecific competition did not significantly impact *A. inexpectatus* progeny at either temperature (10 and 20 °C). With regard to interspecific competition, at 10 °C *A. inexpectatus* was only significantly affected by interspecific competition when it parasitised before *A. nitens* (Table 4.2), resulting in a reduction of 56% offspring per female. Conversely, at 20 °C *A. inexpectatus* was affected by interspecific competition only when it parasitised after *A. nitens*, with an offspring reduction of 65%.

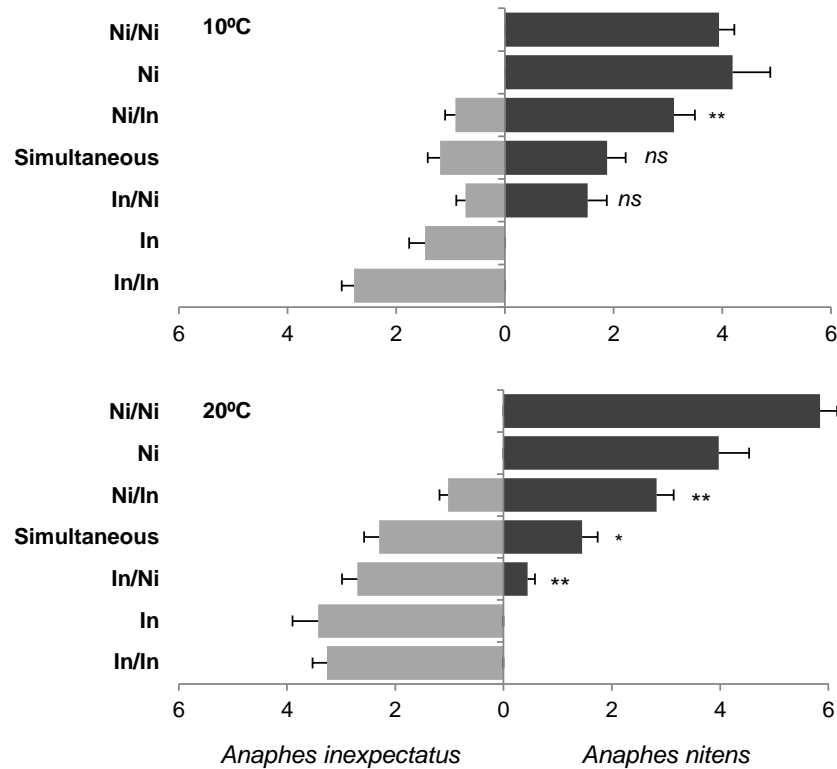


Fig. 4.2 Number of parasitised *Gonipterus platensis* eggs (mean \pm SE) per treatment and per *Anaphes* species, at 10 °C and 20 °C. Each female parasitoid was allowed to parasitise one fresh egg capsule for four hours. Treatments: two *A. nitens* in sequence (Ni/Ni); one *A. nitens* alone (Ni); one *A. nitens* followed by one *A. inexpectatus* (Ni/In); one female of each species at the same time (Simultaneous); one *A. inexpectatus* followed by one *A. nitens* (In/Ni); one *A. inexpectatus* alone (In); and two *A. inexpectatus* in sequence (In/In). Asterisks represent significant differences between species, for each treatment and temperature (Wilcoxon Signed Ranks test: ** $p < 0.001$, * $p < 0.05$, ns not significant).

Table 4.2 Number of fully developed progeny per female (mean \pm SE) in *Anaphes nitens* and *A. inexpectatus*, over different competition treatments, on *Gonipterus platensis* egg capsules. Each female parasitoid was allowed to parasitise one fresh egg capsule for four hours. Treatments: one *A. nitens* alone (Ni); one *A. inexpectatus* alone (In); two *A. nitens* in sequence (Ni/Ni); two *A. inexpectatus* in sequence (In/In); one female of each species at the same time (Simultaneous); one *A. nitens* followed by one *A. inexpectatus* (Ni/In); one *A. inexpectatus* followed by one *A. nitens* (In/Ni).

Temperature	Type	Treatment	n	Number of progeny per female	
				<i>A. nitens</i>	<i>A. inexpectatus</i>
10 °C	No competition	Ni	26	4.19 \pm 0.69 a	-
		In	32	-	2.25 \pm 0.49 ab
	Intraspecific competition	Ni/Ni	67	1.97 \pm 0.14 b	-
		In/In	66	-	2.42 \pm 0.25 a
	Interspecific competition	Simultaneous	58	1.88 \pm 0.34 b	1.64 \pm 0.31 bc
		Ni/In	54	3.11 \pm 0.38 a	1.15 \pm 0.25 bc
		In/Ni	40	1.53 \pm 0.35 b	1.00 \pm 0.27 c
20 °C	No competition	Ni	38	3.97 \pm 0.56 a	-
		In	40	-	4.55 \pm 0.66 a
	Intraspecific competition	Ni/Ni	78	2.92 \pm 0.16 b	-
		In/In	85	-	3.42 \pm 0.32 a
	Interspecific competition	Simultaneous	64	1.45 \pm 0.28 c	3.28 \pm 0.41 a
		Ni/In	80	2.83 \pm 0.32 b	1.59 \pm 0.25 b
		In/Ni	83	0.45 \pm 0.13 d	4.33 \pm 0.50 a

Different letters indicate significant differences between parasitism treatment for each species, at each temperature (GLM, post-hoc LSD, $p < 0.05$).

3.2. Factors affecting host discrimination and the outcome of competition

Evidence of host discrimination between parasitised eggs (PEs) and unparasitised eggs (UEs) was found in both *A. nitens* and *A. inexpectatus* (Table 4.3). *Anaphes nitens* rejected PEs by conspecifics in 18.5% to 66.7% of encounters, while rejection of unparasitised eggs was 5.6% or less. Differences were significant for all intervals between ovipositions. In addition, *A. nitens* discriminated between PEs by *A. inexpectatus* versus UEs for time intervals between ovipositions of one or three days, resulting in rejection rates of 52.2% and 56.5%, respectively. UEs were significantly less rejected, with rejection rates of at most 2.2%. In *A. inexpectatus*, females rejected PEs by conspecifics significantly more than UEs three and six days after the initial parasitism, with rejection rates of 59.1% and 21.9% for PEs and of 2.3% and 3.1% for UEs, respectively. No significant differences between PEs and UEs were found for shorter periods after the initial parasitism (six hours or one day). In addition, *A. inexpectatus* did not significantly discriminate between PEs by *A. nitens* and UEs for any time interval between ovipositions (Table 4.3). In an overall analysis, the likelihood of a female rejecting a PE was significantly affected by the order of parasitism ($W_3= 16.089$, $p= 0.001$) and the time interval between ovipositions ($W_3= 46.333$, $p< 0.001$), and no significant interaction was found between the two factors ($W_9= 13.546$, $p= 0.139$). For all combinations of parasitism order, the lowest rejection rates of PEs were found when the second parasitism occurred six hours after the first (between 2.9% and 18.5%), and the highest rejection rates occurred at a 3-day delay between ovipositions (between 25.8% and 66.7%). The choice of which egg was parasitised first, UE or PE, followed a similar pattern of that of host rejection rates. Overall, whenever females exhibited host discrimination by rejecting PEs significantly more than UEs, they would select UEs first in 65.6% to 78.3% of encounters (Table 4.3).

In both species, females rejected eggs either through antennation (the female would inspect the egg externally with its antennae after which it would walk away) or probing (the female would insert its ovipositor in the host but after internal inspection would withdraw without ovipositing). On average, egg rejection occurred by antennation in 26.8% of encounters and by probing in 73.2% of encounters, but the time between ovipositions significantly affected this ratio ($F_{3,153}= 4.311$, $p= 0.006$). When eggs had been parasitised more recently (six hours), no significant difference

was found between rejection by antennation (55.6% of encounters) or probing (44.4% of encounters). For longer periods, egg rejection by probing was significantly more used, ranging from 69.2% to 85.2% of encounters *versus* 14.8% to 30.8% for antennation. Host inspection by probing was usually fast when the interval between ovipositions was three days or less, with most of the hosts being rejected or parasitised within one minute. In eggs where the first parasitoid had been developing for six days, females frequently displayed long probing behaviour, lasting up to one hour (data not shown).

The outcome of interspecific competition between *A. nitens* and *A. inexpectatus* in individual eggs is shown in Fig. 4.3. In most situations, an interval of 1 day or three days between ovipositions resulted in advantage to the species parasitising first. For the shortest period between ovipositions (under six hours) most of the results were not significant. Conversely, if the period between ovipositions was six days, the species parasitising secondly would usually prevail, except when *A. nitens* parasitised eggs previously parasitised by *A. inexpectatus* multiple times (In+/Ni). When *A. inexpectatus* was allowed to parasitise multiple times, both as first or second parasitising species (In+/Ni and Ni/In+), the probability of outcompeting *A. nitens* increased.

In *A. inexpectatus* and *A. nitens*, significant differences were found in the suitability for development of host eggs of different ages ($F_{7,933} = 9.296$, $p < 0.001$), and no differences were found between species ($F_{1,933} = 0.216$, $p = 0.642$). As shown in Table 4, eggs aged from six hours to three days were equally suitable for immature development in both *Anaphes* species (ca. 90% or more of the parasitised eggs successfully produced progeny). A significant reduction in suitability was found in eggs with six days, to 70.2% and 78.3% viability in *A. inexpectatus* and *A. nitens*, respectively.

Table 4.3 Intra- and interspecific host discrimination between parasitised (PE) and unparasitised (UE) eggs in *Anaphes nitens* and *A. inexpectatus* on *Gonipterus platensis* individual eggs, evaluated as the percentage of rejected eggs and of the first egg selected for parasitism. Treatments: two *A. nitens* in sequence (Ni/Ni); one *A. inexpectatus* followed by one *A. nitens* (In/Ni); two *A. inexpectatus* in sequence (In/In); and one *A. nitens* followed by one *A. inexpectatus* (Ni/In).

Species	Type of competition	Parasitism treatment	Time interval between ovipositions	Rejected eggs (%)			First egg selected for parasitism (%)			n		
				PE	UE	Sig.	PE	UE	Sig.			
<i>A. nitens</i>	Intraspecific	Ni/Ni	6 hours	18.5	0.0	*	51.9	48.1	ns	27		
			1day	35.0	0.0	*	25.0	75.0	*	20		
			3days	66.7	0.0	**	25.9	74.1	**	27		
			6days	44.4	5.6	*	31.6	68.4	ns	19		
	Interspecific	In/Ni	6 hours	2.9	2.9	ns	61.8	38.2	ns	34		
			1day	52.2	0.0	**	26.1	73.9	**	46		
			3days	56.5	2.2	**	21.7	78.3	**	46		
			6 days	22.9	8.6	ns	45.7	54.3	ns	35		
			Intraspecific	In/In	6 hours	3.8	0.0	ns	42.3	57.7	ns	26
					1day	17.9	3.6	ns	57.1	42.9	ns	28
3days	59.1	2.3			**	29.5	70.5	**	44			
6days	21.9	3.1			*	34.4	65.6	*	32			
<i>A. inexpectatus</i>	Interspecific	Ni/In	6 hours	5.9	8.8	ns	58.8	41.2	ns	34		
			1day	12.0	8.0	ns	44.0	56.0	ns	25		
			3days	25.8	9.7	ns	58.1	41.9	ns	31		
			6days	16.7	4.2	ns	62.5	37.5	ns	24		

Asterisks represent significant differences between parasitised egg (PE) and unparasitised egg (UE) within each parasitism treatment and time interval between ovipositions (** $p < 0.001$, * $p < 0.05$, ns not significant), for percentages of rejected eggs (Wilcoxon Signed Ranked test) and first egg selected for parasitism (Binomial distribution test).

The outcome of interspecific competition between *A. nitens* and *A. inexpectatus* in extracted eggs is shown in Fig. 4.3. In most situations, an interval of three days between ovipositions resulted in advantage to the first species that parasitised. For the shortest period between ovipositions (six hours) the results were not significant. Inversely, if the period between ovipositions was six days, the second parasitising species would usually prevail, except when *A. nitens* parasitised eggs previously parasitised by *A. inexpectatus* multiple times (In+/Ni). When *A. inexpectatus* was

allowed to parasitise multiple times, the probability of outcompeting *A. nitens* increased, both as the first or the second parasitising species (In+/Ni and Ni/In+).

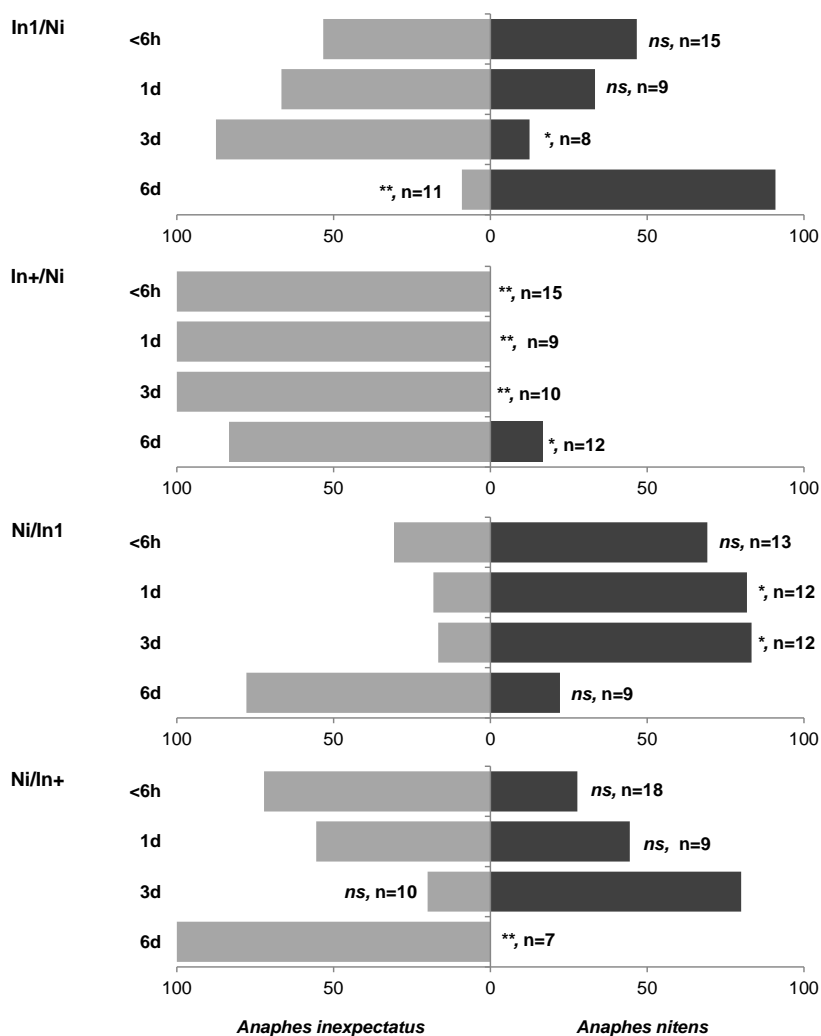


Fig. 4.3 Outcome of interspecific competition between *Anaphes inexpectatus* and *A. nitens* in *Goniopteris platensis* in individual eggs, expressed as the frequency at which each species emerged from each multiparasitised egg in each parasitism treatment. Treatments: one *A. inexpectatus* parasitising once followed by one *A. nitens* (In1/Ni); one *A. inexpectatus* parasitising twice or more followed by one *A. nitens* (In+/Ni); one *A. nitens* followed by one *A. inexpectatus* parasitising once (Ni/In1); and one *A. nitens* followed by one *A. inexpectatus* parasitising twice or more (Ni/In+). Interval between parasitism: less than six hours (<6h); one day (1d); three days (3d); and six days (6d). Asterisks represent significant differences between species for each treatment and interval (χ^2 test, ** $p < 0.001$, * $p < 0.05$, ns not significant).

Table 4.4 Suitability of *Gonipterus platensis* individual eggs and egg capsules of four ages for *Anaphes inexpectatus* and *A. nitens* pre-imaginal development and parasitism (mean \pm SE).

Species	Host age	Pre-imaginal development viability (%)	n
<i>A. inexpectatus</i>	6 hours	97.2 \pm 2.6 a	141
	1 day	94.2 \pm 3.7 a	69
	3 days	89.3 \pm 2.9 a	112
	6 days	70.2 \pm 3.1 b	94
<i>A. nitens</i>	6 hours	91.2 \pm 2.2 a	181
	1 day	94.2 \pm 2.7 a	121
	3 days	91.1 \pm 2.7 a	123
	6 days	78.3 \pm 3.2 b	92

Different letters indicate significant differences between host ages. *Anaphes inexpectatus* and *A. nitens* were analysed separately (GLM with Binomial distribution and post-hoc LSD tests, $p < 0.05$).

4. Discussion

The results of the present study indicate that *A. inexpectatus* and *A. nitens* are likely to compete for the eggs of *G. platensis* both by exploitation (i.e. when one species decreases the amount of resources available to the other species) and interference processes (i.e. when the competing species directly interfere with each other). The outcome of competition between the two parasitoids was affected by the order of parasitism, temperature, the time interval between ovipositions, and the number of eggs laid by the gregarious *A. inexpectatus*.

Parasitising first usually gave parasitoids a competitive advantage, similarly to what has been reported for other parasitoid species (Irvin et al. 2006; Magdaraog et al. 2012). However, temperature mediated the extent to which a species benefited from parasitising first. At 20 °C, the first parasitoid outcompeted the second regardless of species combination, whereas at 10 °C this advantage was only significant when *A. nitens* parasitised first.

More than just the order of parasitism, the interval between sequential ovipositions affected the outcome of competition, as shown in the set of trials using exposed eggs. While individual host eggs are not likely to occur in the field, thus not reflecting

natural conditions, removing the eggs from the egg capsules was a necessary procedure to confirm the occurrence and number of ovipositions in each egg. In these trials, the parasitoid ovipositing first typically succeeded over the second species if the interval between ovipositions was one or three days. For short intervals between ovipositions (under six hours) the advantage of the first species was less pronounced. Six days after parasitism, the larva of the second species usually prevailed over the first. One possible mechanism for this to happen might be that both *A. inexpectatus* and *A. nitens* eliminated their competitors through physical attack by the first instar mymariform larva (direct competition). In genus *Anaphes*, first instar larvae are usually mandibulate and mobile, while the second instar larvae are grub-like and passive (Tooke 1955; van Baaren et al. 1997). Although the larval development of *A. inexpectatus* has not yet been described, it is known that *A. nitens* larvae reach the second instar three to five days after parasitism (Tooke, 1955). Because these species have similar development times from egg to adult (21 days for *A. nitens* and 19 days for *A. inexpectatus*, at 20 °C; Valente et al. 2017b), it is reasonable to assume that their larvae have similar development rates. Therefore, assuming that *A. inexpectatus* first instar larva is also mandibulate, six days after the initial parasitism the first larva would likely have moulted into the passive second instar. This second instar might be more susceptible to attack, thus explaining the success of the species parasitising secondly. An alternative explanation could be that the second parasitoid acted as a facultative hyperparasitoid. Konopka et al. (2017) showed that the egg parasitoid *Trissolcus cultratus* (Mayr) (Hymenoptera: Scelionidae) was able to hyperparasitise *Trissolcus japonicus* (Ashmead) when the larvae of the former species was fully grown. However, if more than one *A. inexpectatus* egg or larva were present inside the host egg at the time of parasitism by *A. nitens*, the former species always outcompeted the latter. When compared to a single oviposition, multiple parasitisms by *A. inexpectatus* as the second parasitising species also improved its odds of surviving in competition with *A. nitens*. Whether this advantage resulted from interference competition (physical attack or hyperparasitism), exploitation competition (increased resource uptake), or a combination of both is unclear. Further studies would be required to clarify the mechanisms involved. Because single parasitism by *A. inexpectatus* was obtained mostly by not allowing females to parasitise more than once, multiple parasitisms would predictably be the most common situation under natural conditions.

Previously parasitised hosts are usually considered low-quality oviposition sites with low return in offspring number or quality, and parasitoid females tend to avoid them (van Alphen and Visser 1990; van Baaren et al. 1995). Acceptance of a previously parasitised host is largely dependent on the female's ability to discriminate between unparasitised and parasitised hosts (host discrimination) and its experience (Ardeh et al. 2005; Harvey et al. 2013). Host discrimination in female parasitoids has been amply demonstrated (Vinson and Iwantsch 1980), and allows the female parasitoid to decide whether or not to oviposit, depending on the circumstances, in order to maximize its reproductive success (van Alphen and Visser 1990; Lebreton et al. 2009). In the present study, *A. nitens* females displayed intra- and interspecific host discrimination towards eggs that had been parasitised one and three days prior by another female. *Anaphes inexpectatus* females discriminated eggs parasitised by conspecifics three and six days after parasitism but willingly accepted hosts parasitised by *A. nitens*, regardless of the time elapsed since parasitism, showing no interspecific host discrimination. Neither *A. nitens* nor *A. inexpectatus* evidenced significant intra- or interspecific host discrimination if parasitism occurred shortly (six hours) after parasitism. The recognition of a previously parasitised host is generally based on marking substances that are placed in and/or on the host during oviposition (van Alphen and Visser 1990). These marks often do not last long, as they are important for the marking parasitoid only during the period when the second clutch could still win in competition for the host (van Alphen and Visser 1990; van Baaren et al. 1994). Our results suggest that females had some ability to identify cues left by previous females through external inspection when the first parasitism had occurred within a few hours. In *Anaphes iole* Girault (Hymenoptera: Mymaridae) females mark the surface of their hosts, and quickly reject previously parasitised eggs by external inspection (Conti et al. 1997). However, external host marking was not seen in *A. nitens* or *A. inexpectatus*, either on naturally laid egg capsules or exposed eggs. Nevertheless, this was not the main recognition mechanism, as eggs parasitised less than six hours before were usually rejected as often as unparasitised eggs. Internal inspection through probing was in most situations required for host rejection to occur. Even though the exact internal cues used by females to identify parasitised hosts remain mostly unknown, they are believed to be chemicals injected by the females (van Baaren et al. 1994; Conti et al. 1997), or produced by the host itself in response to parasitism (Gauthier and Monge 1999). Santolamazza-Carbone et al. (2004) also

showed in *A. nitens* that rejection of previously parasitised egg capsules was more often done after internal inspection than after external inspection. Although host discrimination seemed limited in both species, *A. nitens* appeared to be better at distinguishing and rejecting previously parasitised hosts than *A. inexpectatus*. The ability to discriminate between parasitised and unparasitised eggs is known in several *Anaphes* species (van Baaren et al. 1994, Conti et al. 1997, Santolamazza-Carbone et al. 2004). For example, Van Baaren et al. (1994) found that *A. listronoti* Huber and *A. victus* Huber (Hymenoptera: Mymaridae) had self-, intra-, and interspecific discrimination abilities. *Anaphes listronoti* and *A. victus* exploit the same microhabitat at the same period of the year and are closely related, possibly resulting from relatively recent speciation (van Baaren et al. 1994), which may explain interspecific discrimination. On the other hand, *A. inexpectatus* and *A. nitens* originate from different regions, which may explain limited interspecific discrimination. The apparent lower ability of *A. inexpectatus* to discriminate between parasitised and unparasitised eggs may however be an artefact of their higher willingness to accept previously parasitised eggs, as several immatures of this species can develop inside a host egg. In solitary parasitoids such as *A. nitens*, the presence of supernumerary juveniles frequently results in the death of all but one immature (Lebreton et al. 2009), and this species is therefore likely to suffer highest fitness costs from super- or multiparasitism. In *A. listronoti* and *A. victus*, superparasitism occurred only when females were forced to return to previously rejected parasitised patches (van Baaren et al. 1994). Faced with a seemingly low quality host patch, a female should be prepared to lay a second egg if there is a good chance of winning the subsequent contest. Superparasitism and multiparasitism should therefore be considered adaptive (Hubbard et al. 1987; van Alphen and Visser 1990). In previous studies with *A. nitens*, superparasitism was considered as an adaptive strategy rather than the inability to recognize previously parasitised eggs (Santolamazza-Carbone and Cordero-Rivera 2003; Santolamazza-Carbone et al. 2004), and this may well be the case of *A. inexpectatus* and *A. nitens* in our study.

Age has been shown to affect host quality for parasitoid development, particularly in late stages, close to hatching (Vinson and Iwantsch 1980, Vinson 2010). In the present study, the success of parasitoid development was significantly lower in host eggs that were closer to hatching (six day old) in both *A. inexpectatus* and *A. nitens*,

but not in younger hosts (three days or less). Our results indicate that the age of the egg is not in itself an impediment to parasitoid development, as progeny emerged successfully from hosts close to hatching in over 70% of the parasitised eggs. These findings differ from the results reported in literature on host age preference by *A. nitens*, which show a steep decrease in egg capsule acceptance by females with increasing host age (Williams et al. 1951; Santolamazza-Carbone et al., 2004). In our study we used individual host eggs instead of egg capsules, which might explain these differences.

Theory predicts that long-term coexistence of two species will most likely occur also when interspecific competition is relatively weak compared to intraspecific competition (Godfray 1994). In our study, *A. nitens* displayed a significant reduction in the number of offspring per female both under intra- and interspecific competition, whereas in *A. inexpectatus* interspecific competition was stronger than intraspecific competition. However, tests were conducted under stable artificial conditions. Although competitive exclusion can result from competition between parasitoid species that share a common host (Mills, 2006; Harvey et al. 2013), competing species are often able to coexist through differences in resource use, spatial or temporal partitioning, intermediate disturbance, or differential adaptation to abiotic conditions (DeBach 1965; Godfray 1994; RoCHAT and Gutierrez 2001; Mills 2006; Pekas et al. 2016). In a previous work, Valente et al. (2017b) showed that temperature affects the performance of *A. inexpectatus* and *A. nitens* under laboratory conditions. In that study, net reproductive rates were higher for *A. inexpectatus* at lower temperatures (10 °C and 15 °C), and higher for *A. nitens* at moderate temperatures (20 °C and 25 °C). However, the results of the present study showed that the outcome of competition between *A. nitens* and *A. inexpectatus* is context dependent. Under field conditions, both species will encounter a variety of abiotic conditions throughout the *G. platensis* egg laying periods, that usually occur in late winter/ early spring and in autumn. As such, situations mimicking all of the tested treatments are expected to overlap in complex spatial-temporal mosaics. Resource partitioning based on differential adaptation to abiotic conditions therefore seems like a plausible mechanism for coexistence. One interesting example is that of *Cephalonomia stephanoderis* Betrem and *Prorops nasuta* Waterston (Hymenoptera: Bethylinidae), which attack the same host stage of the coffee borer *Hypothenemus*

hampei (Ferrari) (Coleoptera: Scolytidae). However, higher altitude coffee plantations seem to be more favourable to *P. nasuta*, while low and middle altitude coffee zones are more suitable for *C. stephanoderis* (Infante et al. 2001).

Further experiments (e.g. small field cage trials) could be used to assess competitive interactions between *A. inexpectatus* and *A. nitens* under more natural conditions. Also, extrinsic factors that can affect the interactions between competing parasitoids, such as differences in foraging strategies or dispersal ability, host density effects, or phenological synchronization with the host (Steinberg et al. 1987; Murdoch et al. 1996; De Moraes et al. 1999; Pedersen and Mills 2004; Cusumano et al. 2012) should be further studied. For example, De Moraes et al. (1999) found that *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) was intrinsically inferior in competition with *Microptilis croceipes* (Cresson) (Hymenoptera: Braconidae), but was more effective in detecting and attacking hosts. In addition, small differences in the competing parasitoids' life history traits can have a profound effect on the outcome of competition. On the displacement of California red scale parasitoid *Aphytis lingnanensis* Compere by *A. melinus* DeBach (Hymenoptera: Aphelinidae), Murdoch et al. (1996) concluded that the former species gained a large advantage from its ability to parasitise smaller hosts, thus improving its searching efficiency. Nevertheless, based on the findings from this study, it seems highly unlikely that *A. inexpectatus* would competitively displace the incumbent *A. nitens*. *Anaphes. inexpectatus* should have a better chance of competing with *A. nitens* if its populations are well established. Assuming that the probability of establishment is an increasing function of propagule size and release events, large numbers of *A. inexpectatus* and multiple releases may be necessary for its establishment in areas where *A. nitens* is already present for several decades.

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CHAPTER 5

Environmental risk assessment of the egg parasitoid *Anaphes inexpectatus* for classical biological control of the Eucalyptus snout beetle, *Gonipterus platensis*

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Environmental risk assessment of the egg parasitoid *Anaphes inexpectatus* for classical biological control of the *Eucalyptus* snout beetle, *Gonipterus platensis*

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Abstract

Classical biological control is a valuable tool against invasive pests, but concerns about non-target effects requires risk assessment studies. Potential non-target effects of *Anaphes inexpectatus* Huber and Prinsloo (Hymenoptera: Mymaridae) were assessed for a classical biological control programme against the *Eucalyptus* snout beetle, *Gonipterus platensis* (Marelli) (Coleoptera: Curculionidae). No-choice tests were conducted with 17 non-target species to assess host specificity, including 11 curculionids. In behavioural observations, *A. inexpectatus* showed no interest in any of the non-target species, but two weevil species were parasitised within five days of exposure, although at significantly lower rates than *G. platensis*. In choice tests, only one non-target, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae), was parasitised, at a rate of 0.6%, while 50.0% of *G. platensis* eggs were parasitised. Based on the host specificity test results and the potential host fauna found in the target area, the likelihood of non-target effects resulting from the release of *A. inexpectatus* is considered to be negligible.

Keywords

Curculionidae, Mymaridae, non-target effects, Portugal, Spain.

1. Introduction

Invasive alien insects may cause serious socio-economic hazards as agricultural and forestry pests, or even risks to the environment and to human or animal health (Kenis and Branco 2010). Classical biological control (CBC) is one of the most successful strategies to control invasive exotic species, whose negative impacts are amplified by the absence of natural enemies in the invaded range (Wingfield et al. 2015). The *Eucalyptus* snout beetle, *Gonipterus platensis* (Marelli) (Coleoptera: Curculionidae), meets this criterion. Native to Australia, *G. platensis* has been accidentally introduced in several parts of the world where it became an important pest of eucalypts. Previously known as *Gonipterus scutellatus* Gyllenhal, recent studies have shown this is a group of cryptic species, among which *G. platensis* is the most widely distributed outside Australia, including Southwestern Europe (Portugal and Spain), South and North America, and New Zealand (Mapondera et al. 2012). CBC using the egg parasitoid *Anaphes nitens* (Girault) (Hymenoptera: Mymaridae) has been the main strategy to control *Gonipterus* spp. worldwide. Despite the high success achieved with *A. nitens* in several important regions for eucalypt wood production, only partial control has been attained in some areas in Portugal, Spain, South Africa, and Western Australia (Cordero-Rivera et al. 1999; Tribe 2003; Loch 2008; Reis et al. 2012; Paine et al. 2015).

Attempts to improve CBC of *G. platensis* include searching for additional Australian natural enemies that could be introduced in the affected areas. *Anaphes inexpectatus* Huber and Prinsloo, a Tasmanian parasitic wasp related to *A. nitens*, is one of the few species that has been studied for that purpose. Following a survey for natural enemies of *G. platensis* in Tasmania by C. Valente in 2008, *A. inexpectatus* was imported into Portugal for laboratory studies on its potential against the pest. References about *A. inexpectatus* in literature are scarce, consisting of species description (Huber and Prinsloo 1990), a brief note on its importation into South Africa and a release attempt in Lesotho (Tribe 2003), and studies underway in Portugal (Valente et al. 2017).

Because classical biological control agents (BCAs) are expected to establish permanently, it is crucial to assess the risk of non-target effects before release into the target area. Concerns about biosafety of CBC have raised debate, leading to substantial research on risk assessment and ecological impacts and to the

production of international standards and national regulations on import and release of BCAs. In order to meet biosafety standards, it is recognised that CBC programmes against arthropods must rely on specific predators or relatively host-specific parasitoids (Barratt et al. 2010). Hence, host range evaluation of BCAs is a key element to assess environmental risks (van Lenteren et al. 2006b; McCoy and Frank 2010).

Several sources of information should be considered in host range assessment, including field observations on the native range and where the BCA has been introduced, literature and museum records, as well as physiological, behavioural, and ecological observations and experiments (Sands and Van Driesche 2000; van Lenteren et al. 2006b; Barratt 2011). Host specificity laboratory testing is perhaps the most important aspect of host range assessment and should be a standard practice in pre-release studies (van Lenteren et al. 2006b). During the last two decades a considerable body of literature on host range screening and risk assessment of natural enemies of arthropods was produced (Sands and Van Driesche 2000; Van Driesche and Murray 2004; van Lenteren and Loomans 2006; van Lenteren et al. 2006a, b; Murray et al. 2010). A generally accepted view is that host specificity tests need to be tailored to the target agent in order to prevent false positives (i.e. non-hosts used by the BCA) and false negatives (i.e. valid hosts not attacked by the BCA) (Sands and Van Driesche 2000; Barratt et al. 2010).

Despite the fact that only *Gonipterus* species have been recorded as hosts of *A. inexpectatus*, here we assess its host specificity by testing 17 non-target species present in the target release area, Southwestern Europe (i.e. Portugal and Spain, where *G. platensis* is present). Based on the results and other available information on *A. inexpectatus* and related species, the risk of releasing this parasitoid is discussed.

2. Material and Methods

2.1. Selection of non-target species for host specificity testing

Candidate non-target species for host range testing were selected based on phylogenetic affinity, ecological similarity, and safeguard considerations, according to Kuhlmann et al. (2006). The following criteria were employed in the selection

process: (a) occurrence in the target area; (b) phylogenetic closeness to *G. platensis*; (c) ecological similarity to *G. platensis*; (d) egg casing similarity; (e) conservation status; (f) economic value (beneficial insects); and (g) availability and/or possibility to obtain eggs under artificial conditions, to prevent previous parasitism by other natural enemies (Table 5.1).

More than 1700 Curculionoidea species are known to occur in Portugal and Spain (Alonso-Zarazaga 2013a). *Gonipterus platensis* belongs to the Australo-Pacific tribe Gonipterini, which does not include any species native to Europe (Oberprieler 2010; Mapondera et al. 2012). Nevertheless, an analysis of known Iberian coleopteran fauna and published phylogenetic literature was conducted to identify the species more closely related to *G. platensis*. Tribe Gonipterini is currently placed in subfamily Entiminae (Alonso-Zarazaga 2013b), but some authors remain reluctant to assign the tribe to a subfamily (Oberprieler et al. 2014) as the phylogeny of several taxa among the Curculionidae is not yet resolved (Barratt et al. 2012). According to recent studies (McKenna et al. 2009; Haran et al. 2013; Gillett et al. 2014; Gunter et al. 2016), the tribes phylogenetically closer to the Gonipterini are Hyperini (subfamily Hyperinae), tribes in subfamily Entiminae (especially tribe Sitonini), and probably the tribes in subfamily Cyclominae. Considering this information, 11 weevils were selected, including species from subfamilies Hyperinae and Entiminae, but also other subfamilies, namely Curculioninae, Lixinae, and Baridinae. Some of these species share ecological similarities with *G. platensis*, as they may occur in eucalypts or in the understorey of eucalypt plantations (see Table 5.1). No weevils were selected based on conservation status, since no Curculionidae species are currently considered endangered in either Portugal or Spain (Council of the European Communities 1992). Five weevil species are listed in the Spanish Red List of Invertebrates but were not included in this study because they are rare, have restricted distribution, and their habitat and ecological niche do not overlap *G. platensis* habitat (see Verdú et al. 2011).

Six non-weevils were also selected for testing. *Cryptocephalus rufipes* (Goeze) (Coleoptera: Chrysomelidae), which is associated with *Salix* spp., was chosen as it lays its eggs inside a hard case, resembling *G. platensis* egg capsules. *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) and the ladybirds *Adalia bipunctata* (Linnaeus), *Coccinella septempunctata* Linnaeus, and *Propylea*

quatuordecimpunctata (Linnaeus) (Coleoptera: Coccinellidae) were selected because they are beneficial insects commonly found in Iberian eucalypt plantations. The *Eucalyptus* long horned borer, *Phoracantha semipunctata* (Fabricius) (Coleoptera: Cerambycidae), was selected since it feeds on eucalypts.

2.2. Insect rearing

Anaphes inexpectatus was originally collected in Tasmania (Australia) between 2010 and 2012 and imported into Portugal for studies under quarantine conditions. Its identity was confirmed by J. Huber (Agriculture and Agri-food Canada). Adults used in tests were obtained from populations maintained under laboratory conditions (10 °C, 70-80% RH and 14:10 L:D photoperiod). Newly emerged parasitoids were placed in glass vials (18 mm diameter, 180 mm long) together with *G. platensis* egg capsules and a drop of honey solution (50% in water). Parasitoids were allowed access to host species for one week, after which the egg capsules were incubated in plastic boxes until the new parasitoid generation emerged. Egg capsules were obtained from field collected *G. platensis* adults maintained at 20 °C, 70-80% RH and 14:10 L:D photoperiod.

Adults of non-target species collected for testing were maintained in plastic boxes with perforated lids (1 L) in a climatic chamber (20 °C, 60% RH, 14:10 L:D). In order to obtain eggs for testing, oviposition substrates provided for each species were inspected every working day. Details on specimen origin, rearing and egg collection are available in Table 5.1.

Table 5.1 Non-target species selected for host specificity tests with *Anaphes inexpectatus* (taxonomic classification *sensu* Löbl and Smetana 2013), selection criteria, provenance, common hosts/ prey, food source for laboratory rearing, and oviposition substrate.

Species	Selection criteria	Provenance	Common hosts/ prey	Food source for laboratory rearing	Oviposition substrate
Curculionidae					
Hyperinae					
Hyperini	PC	Organic alfalfa field – Coimbra, PT	<i>Medicago sativa</i> L., <i>Trifolium</i> L. spp., other Leguminosae	Alfalfa (<i>Medicago sativa</i> L.) stems	Alfalfa stems
<i>Hypera postica</i> (Gyllenhal)					
Entiminae					
Sitonini	PC	Organic alfalfa field – Coimbra, PT	<i>Vicia</i> L. spp., <i>Lupinus</i> L. spp., <i>Trifolium</i> L. spp., <i>Medicago</i> L. spp., other Leguminosae	Alfalfa (<i>Medicago sativa</i> L.) stems	Folded glazed paper
<i>Sitona lineatus</i> (Linnaeus)					
Naupactini	PC	<i>Metrosideros</i> Banks ex Gärtner sp. – Aveiro, PT	Polyphagous herbivore	Rose (<i>Rosa</i> L. sp.) leaves	Folded glazed paper
<i>Naupactus cervinus</i> Boheman					
Cneorhinini	PC, ES	<i>Ruscus aculeatus</i> L. – Sever do Vouga, PT	Polyphagous herbivore	Rose (<i>Rosa</i> L. sp.) leaves	Folded glazed paper, rose leaves
<i>Polydus hispanus ludificator</i> (Gyllenhal)					
<i>Polydus hispanus hispanus</i> (Herbst)	PC, ES	<i>Vicia faba</i> L.– Gondomar, PT; <i>Genista</i> L. spp. – Caramulo, PT	Polyphagous herbivore	Rose (<i>Rosa</i> L. sp.) leaves	Folded glazed paper, rose leaves
<i>Philopeton plagiatum</i> (Schaller)	PC, ES ^a	<i>Carpobrotus edulis</i> (L.) N.E. Br – Aveiro, PT	Polyphagous herbivore	Beet (<i>Beta vulgaris</i> L.) leaves	Folded glazed paper, beet leaves
Brachyderini	PC, ES ^b	<i>Quercus suber</i> L.- Soure, PT; <i>Cistus ladanifer</i> L.- Chamusca, PT	Polyphagous herbivore	<i>Cistus ladanifer</i> L. bouquets	Folded glazed paper
<i>Brachyderes lusitanicus</i> (Fabricius)					
Polydrusini	PC, ES	<i>Cistus crispus</i> L., <i>Cistus</i> L. spp. – Pombal, PT; <i>Vicia faba</i> L. – Aveiro, PT	Polyphagous herbivore	<i>Cistus</i> L. spp. stems	Folded glazed paper
<i>Polydrusus pulchellus</i> Stephens					
<i>Polydrusus smaragdulus</i> Fairmaire	PC, ES	<i>Genista</i> L. sp. – Caramulo, PT	Polyphagous herbivore	<i>Cistus</i> L. spp. stems	Folded glazed paper
Curculioninae					
Curculionini	PC	<i>Quercus suber</i> L., <i>Quercus</i> L. spp. – Soure, PT	<i>Quercus</i> L. spp., <i>Castanea sativa</i> Mill.	Semi-peeled <i>Quercus suber</i> L. acorns and diluted honey	Acorns
<i>Curculio elephas</i> (Gyllenhal)					
Lixinae					
Lixini	PC	<i>Vicia faba</i> L. – Gondomar, PT; <i>Malva</i> L. sp. – Aveiro, PT	<i>Malva</i> L. spp., <i>Vicia faba</i> L.	Fava bean (<i>Vicia faba</i> L.) stems	Fava bean stems
<i>Lixus pulverulentus</i> (Scopoli)					
Baridinae					
Baridini	PC	<i>Malva</i> L. sp. – Aveiro, PT	<i>Malva</i> L. spp.	Mallow (<i>Malva</i> L. sp.) stems	Mallow stems
<i>Malvaevora timida</i> (Rossi)					
Cerambycidae					
Cerambycinae					
Phoracanthini	ES	<i>Eucalyptus globulus</i> Labill. – Aveiro, PT	<i>Eucalyptus</i> L'Hér. spp.	Diluted honey	Filter paper
<i>Phoracantha semipunctata</i> (Fabricius)					

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Species	Selection criteria	Provenance	Common hosts/ prey	Food source for laboratory rearing	Oviposition substrate
Chrysomelidae Cryptocephalinae Cryptocephalini <i>Cryptocephalus rufipes</i> (Goeze)	ECS	Eggs provided by Dr. Matthias Schöller (Biologische Beratung, Berlin, Germany)	<i>Salix</i> L. spp.	-	-
Coccinellidae Coccinellinae Coccinellini <i>Adalia bipunctata</i> (Linnaeus)	BI, ES	Larvae purchased from Entocare (The Netherlands)	Generalist predator (mainly aphidophagous)	Aphids (infested bean leaves), diluted honey and pollen	Filter paper
<i>Coccinella septempunctata</i> Linnaeus	BI, ES	<i>Dittrichia viscosa</i> (L.) Greuter – Aveiro, PT	Generalist predator (mainly aphidophagous)	Aphids (infested <i>D. viscosa</i> (L.) Greuter leaves), diluted honey and pollen	Filter paper
<i>Propylea quatuordecimpunctata</i> (Linnaeus)	BI, ES	<i>Dittrichia viscosa</i> (L.) Greuter – Aveiro, PT	Generalist predator (mainly aphidophagous)	Aphids (infested <i>D. viscosa</i> (L.) Greuter leaves), diluted honey and pollen	Filter paper
Chrysopidae Chrysopinae Chrysopini <i>Chrysoperla carnea</i> (Stephens)	BI, ES	Eggs provided by Koppert Biological Systems (The Netherlands)	Generalist predator	-	-

PC – phylogenetic closeness; ES – ecological similarity; ECS – egg casing similarity; BI – beneficial insect; PT – Portugal.

a – found by the authors feeding on *Eucalyptus globulus* Labill; **b** – found by the authors feeding on *E. globulus* × *E. botryoides*.

Table 5.2 *Anaphes inexpectatus* female behaviour (number and percentage of females exhibiting antennation and probing/oviposition) and parasitism (mean proportion \pm SE of parasitised host eggs, number of emerging parasitoid offspring and mean proportion \pm SE of inviable eggs due to probing) in small arena no-choice tests with 17 non-target hosts and *Gonipterus platensis*.

Test species	Number of replicates	Number of host eggs	Female behaviour (Number/ %)		Parasitism		Inviability eggs due to probing (%)
			Antennation	Probing/ oviposition	Parasitised host eggs (%)	Number of emerging <i>Anaphes inexpectatus</i>	
<i>Gonipterus platensis</i>	20	205	15 (75)	9 (45)	41.7 \pm 6.0	21	0
<i>Hypera postica</i>	23	194	7 (30)	0	1.4 \pm 1.1	2	0
<i>Sitona lineatus</i>	16	24	0	0	0	0	0
<i>Naupactus cervinus</i>	24	587	7 (29)	0	0	0	0
<i>Polydus hispanus</i>	58	374	11 (19)	0	0	0	16.8 \pm 4.9
<i>P. hispanus ludificator</i>	30	237	8 (27)	0	0	0	25.7 \pm 8.0
<i>P. hispanus hispanus</i>	28	137	3 (11)	0	0	0	7.1 \pm 5.0
<i>Philopedon plagiatum</i>	13	34	0	0	0	0	0
<i>Brachyderes lusitanicus</i>	15	196	4 (27)	0	16.0 \pm 8.4	8	0
<i>Polydrusus pulchellus</i>	26	1032	0	0	0	0	0
<i>Polydrusus smaragdulus</i>	20	503	1 (5)	0	0	0	0
<i>Curculio elephas</i>	16	20	0	0	0	0	0
<i>Lixus pulverulentus</i>	24	24	0	0	0	0	0
<i>Malvaevora timida</i>	17	17	0	0	0	0	0
<i>Phoracantha semipunctata</i>	28	307	6 (21)	0	0	0	0
<i>Cryptocephalus rufipes</i>	27	216	4 (15)	0	0	0	0
<i>Adalia bipunctata</i>	12	171	0	0	0	0	0
<i>Coccinella septempunctata</i>	24	193	0	0	0	0	0
<i>Propylea quatuordecimpunctata</i>	19	105	1 (5)	0	0	0	0
<i>Chrysoperla carnea</i>	20	160	1 (5)	0	0	0	0

2.3. Host specificity tests

Specificity tests were conducted at 20 °C, 60% RH, 14:10 L:D in a FITOCLIMA 13000 EDTU walk-in chamber. Recently emerged parasitoids (under 48-hour old) and females with no previous parasitising experience were used. Parasitoids were provided with honey solution (50% in water) as food. Non-target host eggs and *G. platensis* egg capsules were collected every day and used until they were at most 48-hour old. After the parasitisation period all eggs were incubated at 20 °C.

Small arena no-choice tests: one female and one male of *A. inexpectatus* were placed in glass vials (16 mm diameter, 100 mm long) and allowed to acclimatise for at least one hour at the assay conditions. Couples were then offered non-target host eggs attached to a white cardstock strip (0.5 × 5.0 cm). Immediately after introducing the eggs in the test vials, behavioural observations of each parasitoid couple were conducted. Behaviour was recorded every three minutes for one hour and categorised as: resting, searching, antennation, and probing/ oviposition. Parasitoids were then allowed to parasitise for five days after which they were removed and the host eggs were incubated. This long exposure period was chosen to maximise acceptance (Browne and Withers 2002). Because larvae of *P. quatuordecimpunctata* eclosed within the five-day testing period at 20 °C, this host was additionally tested at 10 °C. The number of replicates for each species depended on host availability and ranged from twelve to thirty. Twenty positive control replicates with one *A. inexpectatus* couple and one *G. platensis* egg capsule were also performed.

Large arena choice tests: ten under 48 hour-old mated *A. inexpectatus* females were placed in glass vials (16 mm diameter, 100 mm long) and allowed to acclimatise for at least one hour at the assay conditions. They were then released in large arenas consisting of clear acrylic cubic boxes (40 cm edge). Each arena contained a variable number of eggs of the non-target species on a bouquet of cut stems of the corresponding host-plant and four *G. platensis* egg capsules on a bouquet of cut *Eucalyptus globulus* Labill. (Myrtaceae) stems. Bouquet placement was randomised to avoid possible differences in shading, which could influence parasitoid behaviour. Parasitoids were allowed to forage for 24 hours, after which the host eggs were incubated in glass vials. Behavioural observations of parasitoids were conducted as previously described in the first and last hours of the test. Negative controls, in which

the eggs were not exposed to parasitoids, were performed to determine the naturally occurring rate of inviable eggs of the non-target species undergoing testing.

In both tests, adult emergence from parasitised eggs was checked daily and the number of emerging parasitoids was recorded. After no adult emergence was recorded for at least two weeks, the eggs were observed with a stereomicroscope (Leica MZ8) or an optical microscope (Leitz Laborlux 12 ME S) and dissected to determine the number of host larvae, inviable eggs (naturally inviable or inviable via probing), and parasitised eggs (including those from which parasitoids emerged). Host parasitism rates and the percentage of inviable eggs due to female probing were calculated.

2.4. Statistical analysis

To compare non-target species with *G. platensis* in no-choice trials, two dependent variables, the proportion of emerging *A. inexpectatus* and the proportion of parasitised eggs in relation to the number of host eggs available, were analysed using binomial GLM ($p < 0.05$) with Probit link function, considering host species as factor. Binomial distribution was also used to analyse the proportion of parasitised eggs in relation to the total of available eggs between species in choice-tests, as well as the proportion of inviable eggs of non-target species between choice test replicates and negative controls. Wald Chi-square statistic (Wald χ^2) and p values are shown. Results are presented as mean \pm standard error (SE). Frequencies of the behavioural category antennation were tested by Chi-square statistic per host species. All analyses were performed with IBM SPSS Statistics 23 (SPSS, Chicago, IL, USA).

3. Results

3.1. Small arena no-choice tests

During the one hour observation period, external inspection of the eggs through antennation was recorded in nine out of the 17 non-target species offered (Table 5.2). Considering only the species eliciting antennation, this behaviour was more frequently observed with *G. platensis* eggs (75%) than with any of the other species

($\chi^2 = 25.6$, $df = 11$, $p = 0.007$). *Anaphes inexpectatus* females showed no probing or oviposition behaviour towards the eggs of any of the non-target species tested. In contrast, 45% of the females probed and/or oviposited in *G. platensis* within one hour of contact with the eggs. After the five-day exposure period to *A. inexpectatus*, only *G. platensis* and two non-target species [*Brachyderes lusitanicus* (Fabricius) and *Hypera postica* (Gyllenhal)] were successfully parasitised. Significant differences between species were found in the percentage of parasitised eggs (Wald $\chi^2 = 73.9$, $df = 2$; $p < 0.001$) and in the number of eggs leading to viable *A. inexpectatus* progeny (Wald $\chi^2 = 15.4$, $df = 2$, $p < 0.001$). Eggs of *G. platensis* were parasitised at a significantly higher rate (41.7%) than those of *B. lusitanicus* and *H. postica* (16.0 and 1.4%, respectively, $p < 0.001$). In addition, more *A. inexpectatus* progeny emerged from *G. platensis* eggs (21 parasitoids) than from *B. lusitanicus* and *H. postica* eggs (8 and 2 parasitoids, respectively, $p < 0.001$). Although no parasitoids emerged from *Polydus hispanus* (Herbst) eggs, 16.8% of the eggs offered (25.7% in *P. hispanus ludificator* and 7.1% in *P. hispanus hispanus*) were recorded as inviable due to probing. Probing behaviour was not detected during the one-hour behavioural observation period, but females were seen probing the eggs during the remaining testing period, which likely caused inviability. Eggs were further examined and no parasitoid immatures were detected.

3.2. Large arena choice tests

Choice tests were performed with *G. platensis* versus the two non-target hosts successfully parasitised by *A. inexpectatus* in no-choice tests, *B. lusitanicus* and *H. postica*. Parasitoids did not exhibit antennation or probing/ oviposition behaviour in either non-target during observation periods. Inversely, *G. platensis* egg capsules were consistently inspected by female parasitoids (Table 5.3). No parasitism was detected in *B. lusitanicus* and only 0.6% of *H. postica* eggs were parasitised, while parasitism rates in *G. platensis* ranged between 40.7 and 50.0% (Table 5.3). The parasitism rate and the number of successfully emerging parasitoids were significantly lower in *H. postica* than in *G. platensis* (Wald $\chi^2 = 78.5$, $df = 1$, $p < 0.001$). Inviability of non-target species showed no evidence of parasitism and their ratio was not significantly different from the ratio found in negative controls (*H. postica*: Wald $\chi^2 = 0.02$, $df = 1$, $p = 0.879$; *B. lusitanicus*: Wald $\chi^2 = 3.8$, $df = 1$, $p =$

0.051). For *B. lusitanicus* the percentage of inviable eggs was low in choice tests (3.6%) and in negative controls (4.6%), whereas for *H. postica* the percentages were 31.9% in choice tests and 25.9% in negative controls (Table 5.3).

Table 5.3 *Anaphes inexpectatus* female host inspection (expressed as the percentage of replicates in which at least one female displayed antennation, probing or oviposition behaviour), parasitism (mean proportion \pm SE of parasitised host eggs and number of emerging parasitoid offspring) and inviable eggs (mean percentage \pm SE) in large arena choice tests with *Brachyderes lusitanicus* and *Hypera postica* versus *Gonipterus platensis*.

Trial	Number of replicates	Host species	Number of eggs	Female inspection (% of replicates)	Parasitism		% Inviabile eggs
					Parasitised host eggs (%)	Number of emerging <i>Anaphes inexpectatus</i>	
<i>Gonipterus platensis</i> vs. <i>Brachyderes lusitanicus</i>	19	<i>G. platensis</i>	677	84	40.7 \pm 4.7	191	9.7 \pm 1.6
		<i>B. lusitanicus</i>	1145	0	0	0	3.6 \pm 1.3
<i>Gonipterus platensis</i> vs. <i>Hypera postica</i>	14	<i>G. platensis</i>	488	100	50.0 \pm 3.4	148	10.8 \pm 1.1
		<i>H. postica</i>	546	0	0.6 \pm 0.6	6	31.9 \pm 6.1
Negative control	24	<i>B. lusitanicus</i>	1080	-	-	-	4.6 \pm 1.3
	17	<i>H. postica</i>	606	-	-	-	25.9 \pm 3.3

4. Discussion

Because it is impossible to test every potential non-target organism in host-specificity assessment, criteria need to be defined to select candidate species. In the present study, 17 species (including six non-weevils) were selected as possible hosts of *A. inexpectatus*. This number is within the range of 12 to 25 species usually tested in arthropod BC programmes (De Clercq et al. 2011).

No-choice tests were selected for a first tier of host specificity assessment, as they are logistically more convenient than choice tests and can provide accurate information on host use (Murray et al. 2010). These tests were performed in small vials with a long exposure period (five days), whereas in other studies exposure times have ranged from 20 minutes (Gilbert and Morrison 1997) to 72 hours (e.g. Krugner et al. 2008). The longer exposure contributed to increase interaction

between parasitoids and the tested host species, which may have led to false positives, overestimating host range (Browne and Withers 2002; Babendreier et al. 2005). On the other hand, it strengthens confidence in negative results, thus providing a solid rationale for classifying unattacked test species as non-hosts (Van Driesche and Murray 2004). Only two species (*B. lusitanicus* and *H. postica*) among the 17 non-targets were found to be suitable for *A. inexpectatus* development in no-choice tests. Nevertheless, parasitism on both species was much lower than recorded on the natural host, suggesting that they are suboptimal hosts for *A. inexpectatus*. In a third species (*P. hispanus*) *A. inexpectatus* performed probing activities, but no parasitism was detected. Size and shape similarity between *G. platensis* and *P. hispanus* eggs, together with the arenas' spatial restriction and long exposure period, may explain why the parasitoid exhibited probing behaviour. BCAs held in confinement are known to accept suboptimal hosts that would otherwise not be attacked (Sands and Van Driesche 2000). Laboratory testing measures the physiological suitability of non-target species for the candidate BCA, but the physiological host range frequently differs from the ecological host range, defined as the set of species actually used as hosts under natural conditions. This discrepancy is due to several factors that influence host selection in the field, including phenological synchrony, habitat, life history, and learning (Louda et al. 2003). Additionally, even if some non-target species that are attacked in the laboratory are confirmed to be attacked in the field, the magnitude of non-target risks in the laboratory is typically overestimated. For example, laboratory studies on host specificity of the parasitoid *Peristenus digoneutis* Loan (Hymenoptera: Braconidae) showed that all non-target species to which it was exposed were attacked, but levels of field parasitism on those species were lower than predicted by laboratory assays (Haye et al. 2005).

Choice-tests allowed us to verify the results from no-choice tests. Using large arenas for the two species parasitised in no-choice tests, as recommended by van Lenteren et al. (2006b), we were able to confirm that, in the presence of the target host, *G. platensis*, parasitism of both non-targets was residual or non-existing.

Behavioural observations were carried out in both sets of tests, as they improve test interpretation (Babendreier et al. 2005; Barratt 2011). Choice tests with *B. lusitanicus* showed that females of *A. inexpectatus* had no interest in its eggs and no parasitism

was recorded. For *H. postica*, even though low parasitism was detected in both no-choice and choice tests, behavioural observations indicated that *A. inexpectatus* females have no immediate reproductive interest in its eggs. In addition, tests with *H. postica* were performed using exposed eggs rather than how they naturally occur (inside alfalfa stems), likely increasing the chance of parasitism. If attack rates on non-target species are significantly lower than recorded for the target, as occurred in the present study, hazard to non-targets under field conditions is expected to be low (van Lenteren et al. 2006a).

The results from no-choice and choice tests combined suggest that *A. inexpectatus* is mostly host-specific. In fact, while most mymarids are not host-species specific they may be genus-specific (Huber 1986). An example is *A. nitens*, which has been used for CBC of *Gonipterus* species worldwide for nine decades, with no records of ever attacking other host genera. Furthermore, it is widely known that parasitoids commonly locate microhabitats as a reaction to chemical cues from their hosts, host plants and/or herbivore-induced plant volatiles (e.g. Fatouros et al. 2008). Within genus *Anaphes*, one study showed that *A. iole* females locate host eggs through specific volatiles released by host plants damaged by *Lygus hesperus* (Knight) (Hemiptera: Miridae) (Manrique et al. 2005). Although no studies on the response to chemical cues are available for *A. inexpectatus*, it is predictable that the parasitoid will forage using cues from *G. platensis* or from its natural host plant. Weevils belonging to the tribes most related to *G. platensis* usually lay their eggs near or in the soil, among roots, on crevices of plant surfaces, or hidden inside stems, branches and fruits (Leschen and Beutel 2014), making it difficult for a non-specialised parasitoid to find them. Moreover, parasitoids are known to rely on previous experience to find and establish preference for hosts, such as contact with host kairomones upon emergence (Fatouros et al. 2008). Therefore, even if parasitoids encounter non-target hosts, these are less likely accepted than their natural hosts. For the above reasons, while *A. inexpectatus* is physiologically capable of parasitising non-target hosts, we conclude that parasitism is unlikely to occur under natural conditions.

This work focuses on the possible impact of *A. inexpectatus* releases on non-target host species. Further concerns regarding direct and indirect effects of BCA introductions include competitive interactions with other organisms, such as

displacement of other natural enemies and indirect effects on the same or other trophic levels (van Lenteren et al. 2006a; Barratt 2011). Although competition between *A. inexpectatus* and the already established *A. nitens* has not been studied, it may occur. Because both species are exotic, the outcome should not be of any consequence to the native fauna, although it could interfere with the success of *G. platensis* control. In addition, there are concerns about hybridisation between introduced BCAs and native natural enemy species, which might cause negative impacts (van Lenteren et al. 2006a; Hopper et al. 2006). However, to the best of our knowledge only two cases of hybridisation between introduced and native parasitoids are described: two species of *Torymus* (Hymenoptera: Torymidae) can hybridise in the field (Yara et al., 2010) and two species of *Diadegma* (Hymenoptera: Ichneumonidae) hybridise under laboratory conditions (Davies et al. 2009). In both cases the introduced and the native parasitoid share a common host, increasing the probability of species encounter.

From our results, we conclude that the overall risk of negative environmental impacts due to the introduction of *A. inexpectatus* in the target area is minimal. Based on the available data, *A. inexpectatus* is not expected to attack non-target hosts in natural conditions and no other negative effects on native species are expected to occur. While defining how much risk is acceptable is probably the most difficult question in risk assessment (McCoy and Frank 2010), decisions should be based on the global risk/ benefit analysis of BCA introduction (van Lenteren and Loomans 2006). In this sense, taking into account the high economic impact of *G. platensis* attacks on eucalypts (Reis et al. 2012), the potential benefits of *A. inexpectatus* clearly surpass any slight risk that the introduction of this parasitoid may pose. If the parasitoid is released and establishes successfully, laboratory predictions on the impacts on both target and non-target species should be further confirmed by performing post-release field studies (Hajek et al. 2016). Nevertheless, post-release monitoring of non-target species may be meaningful only several years after releasing the BCA (Froud and Stevens 2004).

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General Conclusions



Gonipterus platensis larvae unparasitised.



Gonipterus sp. larva parasitised by *Entedon magnificus*.



Gonipterus sp. larva parasitised by *Oxyserphus* sp..



Gonipterus sp. larva parasitised by *Anagonia* sp.
(photos by C. Gonçalves).

General Conclusions

The snout beetle *G. platensis* is the main pest of *Eucalyptus* spp. plantations in Portugal, similarly to what occurs in other regions in the world. The present study confirmed the high economic importance of this defoliator, by assessing its impact on *E. globulus* wood production in Portugal during a 20-year period. According to the results, defoliation by *G. platensis* resulted in wood losses of 648M euros in the study area over the past 20 years. Such economic losses happened in spite of partial success of biological control by *A. nitens*. However, without biological control, losses would predictably have been from almost four times higher, for a scenario where *G. platensis* populations were controlled exclusively with insecticides, to eleven times higher if wood losses were offset by imported wood. These results show that CBC can have a high economic benefit, even if only partial control of the target pest is achieved.

CBC has been an important strategy to deal with non-native insect pests in forests. According to a literature review included in this study, at least 37 Australian natural enemies have been used as biological control agents against eucalypt pests, and about half of these were successful. Compared with other management methods, like the use of insecticides, biological control has several advantages, as it is harmless to humans, safer to the environment, provides a long-term solution for pest problems, and is generally more cost-effective. Still, very few studies address the economic impact of biological control strategies in forestry. In this work, it was demonstrated that the anticipation of biological control by a few years can produce significant gains. By assessing the costs and the benefits of the CBC programme intended to accelerate the establishment of *A. nitens* in Portugal, a benefit-cost ratio ranging from 67 to 347 was found, if the benefits of releasing *A. nitens* were considered to have occurred for one or three years, respectively. However, since the calculations were based exclusively on the impact on wood production and nonmarket values were not accounted for, the benefits of the programme were likely underestimated.

Because *A. nitens* has not provided complete control of *G. platensis*, field surveys aiming to find other natural enemies that could be used as classical biological control agents were carried out in Tasmania, Australia. These surveys allowed the identification of three larval parasitoids (*E. magnificus*, *Oxyserphus* sp., and *Anagonia* sp.) and five egg parasitoids (*A. tasmaniae*, *A. inexpectatus*, *C. damoni*,

Cirrospilus sp., and *Euderus* sp.). This work also accounts for the first report of *A. nitens* in Tasmania, and the available information suggests that this species was recently introduced from the Australian mainland, from where it is apparently native. In a first attempt to rear the natural enemies found in Tasmania, the egg parasitoid *A. inexpectatus* was the only species providing stable laboratory populations and therefore it was selected for further studies. By comparing the life history traits of *A. inexpectatus* and *A. nitens* at six temperatures (5, 10, 15, 20, 25, and 30 °C), the range from 10 °C to 20 °C was found to be the most adequate for immature development in both species. Lower development thresholds were similar between both parasitoids, with 6.0 °C recorded for *A. inexpectatus* and 5.4 °C for *A. nitens*. Despite these similarities, relevant differences were found between the two species. Net reproductive rates were higher for *A. inexpectatus* at lower temperatures (10 °C and 15 °C), and higher for *A. nitens* at moderate temperatures (20 °C and 25 °C). *Anaphes inexpectatus* evidenced higher tolerance than *A. nitens* to the highest temperature tested (30 °C).

Temperature also affected the outcome of competitive interactions between the two parasitoid species, with advantage to *A. inexpectatus* at 20 °C. However, at both temperatures tested (10 °C and 20 °C), when *A. nitens* parasitised first it was able to outcompete *A. inexpectatus*. When *A. inexpectatus* parasitised an egg multiple times, its competitive ability against *A. nitens* increased, regardless of the interval between ovipositions (from less than six hours to six days). Both species were able to parasitise eggs 6-hour to 6-day old, but the proportion of viable parasitoids developing in host eggs decreased in 6-day old eggs. According to the results of competition experiments, *A. inexpectatus* will not displace *A. nitens* and the two species should be able to coexist in field conditions. However, *A. inexpectatus* establishment in the field in areas where *A. nitens* is already present may be delayed or even prevented due to interspecific competition. Therefore, multiple field releases and large numbers of *A. inexpectatus* per release may be necessary for establishment.

The introduction of new biological control agents must be preceded by studies that demonstrate not only their potential efficacy and suitability for the climatic conditions of the introduced area, but also the absence of environmental risks. In this study, the environmental risk assessment for releasing *A. inexpectatus* in the Iberian Peninsula

was based mostly on the potential host fauna found in the target area and on laboratory choice and no-choice host specificity tests, involving 17 non-target species. This risk assessment suggests that *A. inexpectatus* is not expected to affect non-target organisms in natural conditions, thus being a safe organism.

Overall, our results contribute to improve the knowledge on biological control in forest ecosystems, its economic benefits and constraints. For the particular case of CBC of *G. platensis*, this work revealed that *A. inexpectatus* may complement the biological control already exerted by *A. nitens*, without displacing the incumbent parasitoid and without affecting non-target species. Considering the high economic importance of *G. platensis* and the potential benefits of classical biological control, *A. inexpectatus* should therefore be tested in field conditions. Additionally, other natural enemies should be further studied as well. Larval parasitoids, in particular, are interesting candidates for a biological control programme against *G. platensis*, since they attack a development stage that is currently free of natural enemies outside the pest's native range.