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When natives go wild...

*Why do some insect species become invasive in their native range?*

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy at Lincoln University by Marie-Caroline Lefort

Lincoln University 2013
Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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Why do some insect species become invasive in their native range?

by

Marie-Caroline Lefort

Since the inception of invasion ecology as a modern field of research, there have been numerous attempts to establish why and how some species become invasive. To date, research emphasis has been almost entirely on exotic species introduced into new areas. As a result, most of the established theories relative to invasion success are not applicable for species that become invasive in their native range. The study of such species can, therefore, offer new directions for the search for mechanisms and the characterisation of traits associated with invasion success. This thesis aimed at investigate some alternative hypotheses using the New Zealand native species *Costelytra zealandica* (White) and *C. brunneum* (Broun) (Scarabaeidae: Melolonthinae) as an invasive and non-invasive congeneric species model.

For many years, the larvae of *C. zealandica* and *C. brunneum* have been considered cryptic. To progress the research in this thesis, two novel molecular methods, based on the use of frass and larval exuviae as non-invasive sources of DNA, were developed to differentiate them. In addition, a detailed comparative taxonomic assessment between *C. zealandica* and *C. brunneum* revealed that three morphological characters allowed accurate identification of third instar larva. For live larvae, especially in field conditions, the morphology of the septula of the raster is usually sufficient to differentiate the two species.

Several comparative studies that determined the feeding preferences and fitness performance of the model species when fed with native and exotic host plants were performed. The results of these experiments supported the existence of strong intra-specific variations in the diet of *C.
zealandica, suggesting the occurrence of evolutionary processes (i.e. host-shift or host range expansion) and the existence of a pre-existing ability to use ‘new’ hosts in this invasive species.

The processes of host-shift and host range expansion were subsequently investigated in C. zealandica, using tussock and white clover as example of native and exotic host plants, respectively. The comparison of fitness response of several populations of this species to various feeding treatments, comprising an artificial host-shift, revealed that C. zealandica populations occurring in exotic pastures have experienced an ecological host-shift rather than just a host-range expansion. Furthermore, the results of this study suggested the existence of distinct host-races in this species.

Additionally, further experiments were performed to determine whether or not C. zealandica became a successful invader through a pre-existing capacity to tolerate the detrimental effects of its new host plant’s defence chemicals. The comparison of the fitness response of C. zealandica and C. brunneum to host defences, artificially triggered and enhanced by the phytohormone jasmonic acid, suggested the existence of a pre-existing ability in the invasive species to tolerate and benefit from the defence chemicals of its exotic host plant.

Finally, the environmental tolerance of C. zealandica and C. brunneum was compared through their survival and growth responses to different temperature regimes, and an attempt was made to relate these fitness responses to the phosphoglucose-6-isomerase (PGI) enzyme system. The invasive species C. zealandica was found to be more tolerant to challenging temperatures than its congener. However, no relationship was observed between the PGI enzyme system and the individual fitness performance of C. zealandica or C. brunneum under various temperature regimes.

This thesis presents new insights into the mechanisms that may have led C. zealandica to reach the status of invader in its native range. The combined results presented in this thesis highlights, in particular, the importance that phenotypic plasticity might have played in the invasion success of C. zealandica. The conclusions raised here offer new research directions for the investigation of the invasion process in phytophagous insects in general.

Keywords: Costelytra zealandica, Costelytra brunneum, Scarabaeidae, larvae, pest, native invader, invasive species, cryptic life-stages, DNA barcoding, COI, frass, exuviae, scarab taxonomy, invasion key-trait,s plant defences, jasmonic acid, belowground induced defences, pre-adaptation, insect-plant interactions, feeding preference, fitness performance, host-shift, host-range expansion, host-race, biotype, phosphoglucose isomerase, thermal tolerance, phenotypic plasticity
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Chapter 1

General introduction

1.1. Thesis context

“When natives go wild…” While you were reading and wondering about the title of this thesis, dozens, if not hundreds, of species, whether they are animals, plants or microorganisms, have somewhere reached the status of potential biological invaders (Simberloff & Rejmanek 2011). Such a process is facilitated by the existence of new biota rearrangements, which can either be geographical or ecological, and may or not involve human intervention (Mack et al. 2000). Hence, by encountering new living conditions some species will quickly spread and reach damaging and persistent population densities; in other words become “pests”. It is widely acknowledged that biological invasions are a global issue and over the past decades have attracted much fundamental and applied research (Richardson 2010). The reasons for such a keen interest are numerous and diverse. Understanding the invasion process and ways to alleviate its impacts, stimulate the research interests of biologists, ecologists, evolutionists, geneticists and even economists. As in most research fields, in invasion biology ‘why’ should be one, if not the most, fundamental question raised, but surprisingly appears to be much less considered than expected. Only a few research articles report fundamental investigations about the essence and the mechanisms underpinning the phenomenon of biological invasions (e.g. Robert 1990, Mack et al. 2000, Keane & Crawley 2002, Callaway & Ridenour 2004). The present thesis explores an as yet not investigated, ‘why’ question that promises to offer new insights about phytophagous insect invasions: “...why do some insect species become invasive in their native range?”.

1.2. Ecological aspect of biological invasions

Invasion ecology is often regarded as a modern field of research that emerged from various older sub-disciplines of ecology such as agriculture, forestry, zoology, botany, pathology and entomology (Lockwood et al. 2007). Even though invasive species were mentioned in several manuscripts dated from the 19th century (Richardson & Pysek 2008), the foundation of this
science is often attributed to Charles S. Elton (1958) and his monograph titled *The Ecology of Invasions by Animals and Plants*.

Richardson (2010) suggested that the rapid growth in popularity of invasion ecology is probably based on the realisation that invasive species increasingly threaten global biodiversity and ecosystem functioning. Richardson (2010) also recognised the potential for unique insights presented by this field of research into biogeography and ecology. Similarly, and even prior to Elton’s book, it was suggested that biological invasions represent novel and - to some degree-natural experiments that contribute to better understanding of ecological and evolutionary processes and of ecology in general (Cadotte et al. 2006), despite the fact that invasion ecology was and is still sometimes dissociated from the rest of ecological disciplines (Davis et al. 2001).

Based on contemporary literature about invasion ecology (i.e. Davis 2006, Mack et al. 2000 and Richardson & Pysek 2006), Richardson and Pisek (2008) defined this science as the study of the human-mediated introduction of organisms in areas outside their potential range that is characterised and restrained by their natural dispersal mechanisms and biogeographical barriers. This definition implies that the organism in question must be an alien species and fails to consider a substantial number of biological invaders. A fairly different position toward the concept of invasion ecology is observed in this thesis, by considering the often forgotten native invaders.

### 1.3. Biological invasion and the question of native invasive species

#### 1.3.1. A problematic terminology

Although the topic of biological invasion has been thoroughly documented (for a review see Richardson 2010), there is some level of ambiguity around the definition of an “invasive species” as indicated by numerous peer review articles debating this subject (e.g. Valéry et al. 2008a, 2009, Colautti & Richardson 2008). This ambiguity seems to also indicate a lack of strong theoretical understanding. This type of uncertainty has been largely discussed recently, because lexical terminology can have a strong impact on scientific communication. As discussed by Heink and Kowarik (2010) in a recent review, clarification of ambiguous terms in ecology is not a recent issue. Indeed, since Murphy and Noon (1991) called for clear operational definitions in biology and ecology, numerous authors attempted to disentangle the established ambiguities regarding the definition of an invasive species (e.g. Davis & Thompson 2000, Collauti & Maclsaac 2004, Collauti & Richardson 2008, Valéry et al. 2008a, 2009). Many terms belonging to the invasion lexicon are, to some extent, inter-connected. The word “pest” for instance, implies invasiveness and reciprocally an invasive species is qualified as pest whenever it becomes detrimental to human or to their concerns (Bos & Parlevliet 1995). Such sort of interconnection may lead to a
cascading misuse of terminology and to an exponential level of ambiguity surrounding the definition of an invasive species.

For many years, the term “invasive” has been more often used as a taxonomic description, that characterised a nuisance species, rather than to describe an ecological phenomenon (Colautti & MacIsaac 2004). As a result, invasive species are most often seen as unwanted species that have been introduced into new areas outside of their native geographical range. The majority of terms employed to qualify invasive species in the literature also reflects this trend (e.g. ‘alien’ (Crawley et al. 1996), ‘exotic’ (Green 1997), ‘non-indigenous’ (Mack et al. 2000; Pimentel et al. 2000; Kolar & Lodge 2001), ‘imported’ (Williamson & Fitter 1996), ‘non-native’ (Davis et al. 2000), ‘immigrant’ (Bazzaz 1986) etc. (see Colautti & MacIsaac 2004 for an exhaustive list). Very few of those terms refer or can be applied to native and endemic species in their native range.

1.3.2. The concept of “native invaders”

As recently debated by Valéry et al. (2009) in a letter in Trends in Ecology and Evolution, ‘invasive species can also be native’. As a general rule, insects are labeled as “pests” and “invaders” once they have passed through three defined stages (1) introduction, (2) establishment of a viable population, and (3) generation of economic and/or environmental harm (Worner 2002). If in the first stage of the above process, the biogeographical criterion (e.g. overcoming major geographical barriers and/or distances) is deliberately overlooked then this schema can also be applied to endemic and native species (Valéry et al. 2008b). The concept of “native invaders” is still greatly debated among invasion biologists and ecologists, despite the fact that Charles Elton (1927) himself employed the term invasion to refer to the rapid spread of native species into a ‘new habitat’ (Simberloff & Rejmanek 2011). Here, ‘new habitat’ shall be perceived and defined as a major exogenous modification of the environment (Valéry et al. 2008b) mostly driven and mediated by human intervention, including eutrophication, habitat and land use changes, urbanisation, loss or addition of predator(s) and climate change (Valéry et al. 2008a, 2008b, 2009, Kobayashi et al. 2011, Carey et al. 2012).

Several attempts have been made to define a “native invader”, and the consensus for a shortened and simplified definition of the term might be ‘species that have become invasive in their own native range’ (Simberloff & Rejmanek 2011, Carey et al. 2012), while a more explicit and thorough definition will be adopted in this thesis as the one proposed by Valéry et al. (2008b) ‘a species already native to (i.e., already an integral part of) the region or the ecosystem it is going to invade, but whose distribution changes owing to a change in its environment’.

Based on the latter definition and because of the unique geographical and historical situation of remote islands, which have resulted in the introduction of numerous plant species (Tye 2006), it is
likely that these islands may be prone to the successful rise of native invaders. For instance, New Zealand, which has seen a large range of agricultural and horticultural plants introduced in the country over the past decades (Esler 1987) and which has been subject to numerous changes in land use after European settlement (Lee et al. 2006), has more than 60 native insect species across 36 different families that have reached the status of pest / invasive species (Appendix 1). By looking at the extent of the phenomenon and the numerous threats that “native invaders” represent locally, scientists are urged to investigate and predict why, when, where and how a native species will transition into an invader within its own native range (Carey et al. 2012).

1.3.3. The imbalance between exotic and native invader research

Biological invasion represents a broad area of research, where numerous scientific sub-disciplines (e.g. ecology, computational biology, genetic, evolution, physiology, molecular biology, biochemistry, ethology etc.) and their techniques are employed to investigate a wide range of organisms across all six kingdoms. Such expansive investigation can occasionally promote confusion, controversy, and unintentional bias, especially if not considered in integrated studies, such that specific areas of potentially productive research can sometimes be overlooked. Within the realm of insects, the biology, ecology and the impact of invasive species are well studied throughout the world. A simple keyword search for the term association insect and invasi* on the Web of KnowledgeSM, between 2000 and 2013, reveals about 12,900 results, which represents about 16% of all publications on insect ecology over this period (based on a similar term association search for insect and ecolog*). Nevertheless, a short meta-analysis carried out in this thesis revealed a significant gap between the number of studies relative to exotic insect invaders and those relative to native ones (Appendix 2). Based on a sample of three representative journals dealing with insect invasions, it appeared that most focus was on exotic organisms invading new regions. In comparison, native invasive insect species have, surprisingly, received almost no attention (Appendix 2), although such imbalance may possibly rely on the misuse of the term invasive species, as discussed earlier. The research reported in this thesis offers to reverse the trend by investigating native insect invaders.

1.3.4. Theoretical invasion mechanisms and native invaders

The potential key traits and explanations why a species may become invasive are extremely diverse. These traits and mechanisms are often referred to as “theories” or “invader characteristics”. When discussing “invader characteristics”, some distinction must be made between the intrinsic characteristics of a species such as dispersal ability (Rejmanek & Richardson 1996), tolerance limits (Jiménez-Valverde & Lobo 2011) and phenotypic plasticity
(Price et al. 2003), and the various external and/or environmental factors that can contribute to the same species to become invasive such as theories associated with bottom-up and top-down controls.

Table 1.1. Non-exhaustive list and short description of theories and key traits established to characterise invasive species and their applicability to native insect invaders.

<table>
<thead>
<tr>
<th>Invasion theory key trait</th>
<th>Species intrinsic characteristic</th>
<th>External or environmental factors</th>
<th>Description</th>
<th>Applicable to native insect species</th>
<th>Reason(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coexistence model</td>
<td>✓</td>
<td></td>
<td>A potential invader can access resources that remain unused by a resident species that has reached its maximum local population size (Fridlay 2011)</td>
<td>Partially</td>
<td>Only when the invaded habitat corresponds to a human-altered environment (e.g. private garden, agricultural field, reservoirs...) constituted by at least one new exotic host plant</td>
</tr>
<tr>
<td>Dispersal ability</td>
<td>✓</td>
<td></td>
<td>Deliberate or involuntary relocation of individuals of a species from a place of origin to another location (McDowall 2011)</td>
<td>No</td>
<td>The «introduction» stage cannot apply to a native invasive species, which is by definition already present in the invaded geographical area</td>
</tr>
<tr>
<td>Empty niches hypothesis</td>
<td>✓</td>
<td></td>
<td>Native species may leave empty niches available for colonisation by another species or be outcompeted by exotic species that have the ability to convert resources into offspring more efficiently (Fridlay 2011)</td>
<td>Partially</td>
<td>Only when the invaded niche corresponds to a human-altered environment (e.g. private garden, agricultural field, reservoirs...) constituted by at least one new exotic host plant</td>
</tr>
<tr>
<td>Enemy release hypothesis</td>
<td>✓</td>
<td></td>
<td>Following the introduction into a new region, a species experiences a decrease in regulation by natural enemies, resulting in a rapid increase in distribution and abundance (Keane and Crawley 2002)</td>
<td>Partially</td>
<td>Natives species and their natural enemies are likely to present a similar geographical distribution in their native range, hence not allowing a complete escape of the invader from its enemies</td>
</tr>
<tr>
<td>Invasion meltdown</td>
<td>✓</td>
<td></td>
<td>Reciprocal establishment facilitation of two or more exotic species that may lead to accelerated impacts on the invaded native ecosystem (Simberloff and Von Holle 1999)</td>
<td>No</td>
<td>Only applicable to exotic species by definition</td>
</tr>
<tr>
<td>Novel weapon hypothesis</td>
<td>✓</td>
<td></td>
<td>Some exotic species may become invasive because they produce novel biochemical weapons, different from the ones of the species of the invaded community, which provides exotics with advantages against their native competitors (Callaway and Ridenour 2004)</td>
<td>No</td>
<td>Only applicable to invasive plant species</td>
</tr>
<tr>
<td>Phenotypic plasticity</td>
<td>✓</td>
<td></td>
<td>Ability of an organism to change and adapt its phenotype in response to changes in the environment (Price et al. 2003)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Propagule pressure</td>
<td>✓</td>
<td></td>
<td>Factor determining whether a species introduced to a new region is likely to be established, based on the number of individuals arriving in the region to which they are not native (Duncan 2011)</td>
<td>No</td>
<td>The «introduction» stage cannot apply to a native invasive species, which is by definition already present in the invaded geographical area</td>
</tr>
<tr>
<td>Tolerance limits</td>
<td>✓</td>
<td></td>
<td>Ecophysiological constraints that restrict species to environmental bounds beyond which they cannot survive (Jiménez-Valverde and Lobo 2011)</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

Although theoretical invasion mechanisms and hypotheses about why species become invasive appear very well researched (Table 1.1), most theories are not as tenable when native insect invaders are considered. First of all, some theories are applicable only to plant species (e.g. novel
weapon hypothesis (NWH) (Callaway & Ridenour 2004) and others, which were initially applied to plant species, were eventually extended to animal species (e.g. dispersal ability (Rejmanek & Richardson 1996), enemies release hypothesis (ERH) (Elton 1958)). Furthermore, a review of several established theories, mechanisms and key traits invoked to explain biological invasions shows that many of them cannot be applied to native species (Table 1.1). Phenotypic plasticity and tolerance limits, which include the thermal tolerance of a species (Jiménez-Valverde and Lobo 2011), appear to be two intrinsic characteristics that are likely to characterise invasiness in native insects (Table 1.1). Therefore, the present thesis aims to investigate these two traits and to identify other potential intrinsic characteristics and external factors that fit well and can readily be applied to native insect invaders by using scarabs as a species model.

1.4. Pest scarabs as species model

1.4.1. General description

Among the Order Coleoptera, the family of Scarabaeidae includes over 31,000 described species (Thakare et al. 2012) and is often considered as one of the most diverse and widely distributed group of beetles (Chandra et al. 2012). The adult life-stage of these insects is often particularly noticeable because of its wide range of stunning and bright colors and ornamentations and its relatively large size (Jackson & Klein 2006, Chandra et al. 2012). On the other hand, the C-shaped yellowish body of the various larval stages of these beetles (Capinera 2008) is less striking and is morphologically more homogeneous amongst species. Nevertheless, it is frequently from the larval form of the scarab from which the common names arise, such as ‘white grubs’, ‘grass grubs’, ‘sugar-cane grubs’, ‘scarab grubs’, ‘root-feeding grubs’ or ‘curl grubs’. Fascination with scarabs is as old as the 1st Egyptian Dynasty (Jackson & Klein 2006), when these insects were regarded as symbol of rebirth. Nowadays, this fascination has become diametrically opposed, particularly when these species are associated with agricultural and productive lands. In such cases, scarabs are not seen as ‘sacred’ anymore, but as threatening invasive pest species.

1.4.2. Scarab larvae as pests

Scarab beetles are mainly phytophagous insects that present a wide variety of feeding habits with respect to the part of the plant they fed on (Jackson & Klein 2006) and as dictated by their life-stages. While adults often feed on the aboveground parts of the plants, scarab larvae are generally root feeders (Cowles et al. 2005) and the consequent damage caused to the roots
means they are frequently regarded as pests (Blossey & Hunt-Joshi 2003; Dittrich-Schröder et al. 2009). They are also usually generalist feeders, so scarab larval outbreaks can affect numerous economically important pasture, agricultural, forestry and horticultural plants (McPeak et al. 2006, Romero-López et al. 2010) on virtually all continents (Jackson & Klein 2006) (Table 1.2).

Even though no scarab species is currently recorded in the top 100 worst invasive alien species in the world on the Global Invasive Species Database (GISD) (Browne & Poorter 2005) that includes 14 insect species, scarab pests are undoubtedly a recurrent and long-term problem. For instance, the Japanese beetle *Popillia japonica* Newman is often characterised as the most widespread and destructive insect of the east of the United States (Potter & Held 2002), despite over a century of research and development of management and control programs (Potter & Held 2002, Jackson& Klein 2006).

A few years ago and for the first time, Jackson and Klein (2006) clearly categorised two types of scarab pests (Table 1.2). The first type comprises exotic and introduced species, and the second, which fits Valéry’s definition of a native invader (2008b), comprises endemic or native scarabs that have reached the status of pest in their home range. The latter type represents between one and two percent of the total number of species described in the family (Jackson& Klein 2006). Among them is the common grass grub, *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae) (Table 1.2), which quickly invaded newly developed European-style pastoral ecosystems in its native home range, causing substantial damage to introduced plant species and rapidly becoming one of New Zealand’s worst pests (Pottinger 1975, Richards et al. 1997).
Table 1.2. Examples of native and exotic invasive scarab species and their invaded range. Note that some of the native invaders presented in this table have also reached the status of exotic invasives outside of their native geographical range and reciprocally, some of the exotic species are also considered as invasive in their home-range. (Adapted from Jackson and Klein 2006).

<table>
<thead>
<tr>
<th>Species</th>
<th>Invader status</th>
<th>Invaded region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adoretus sinicus Burm.</td>
<td>introduced/exotic invader</td>
<td>Hawaii</td>
</tr>
<tr>
<td>Adoretus versutus Harold</td>
<td>introduced/exotic invader</td>
<td>Samoa</td>
</tr>
<tr>
<td>Adoretus versutus Harold</td>
<td>introduced/exotic invader</td>
<td>Vanuatu, Futuna Islands</td>
</tr>
<tr>
<td>Adoryphorus couloni Burmeister</td>
<td>introduced/exotic invader</td>
<td>New Zealand</td>
</tr>
<tr>
<td>Amphilimon majalis (Razoum)</td>
<td>introduced/exotic invader</td>
<td>North America</td>
</tr>
<tr>
<td>Anomaia orientalis Waterhouse</td>
<td>introduced/exotic invader</td>
<td>North America</td>
</tr>
<tr>
<td>Aphodius tasmaniae Hope</td>
<td>introduced/exotic invader</td>
<td>New Zealand</td>
</tr>
<tr>
<td>Cyclocephala signaticollis Burmeister</td>
<td>introduced/exotic invader</td>
<td>Australia</td>
</tr>
<tr>
<td>Hoplochelus marginalis (Fairmaire)</td>
<td>introduced/exotic invader</td>
<td>Reunion Island</td>
</tr>
<tr>
<td>Maladera castanea (Arrow)</td>
<td>introduced/exotic invader</td>
<td>North America</td>
</tr>
<tr>
<td>Maladera matrida Argaman</td>
<td>introduced/exotic invader</td>
<td>Israel, Saudi Arabia</td>
</tr>
<tr>
<td>Papuana unidolis Prell</td>
<td>introduced/exotic invader</td>
<td>Fiji, New Caledonia</td>
</tr>
<tr>
<td>Popillia japonica Newman</td>
<td>introduced/exotic invader</td>
<td>North America, Açores</td>
</tr>
<tr>
<td>Protasta pryeri (Jenseni)</td>
<td>introduced/exotic invader</td>
<td>Midway</td>
</tr>
<tr>
<td>Adoryphorus couloni (Burmeister)</td>
<td>native invader</td>
<td>Australia</td>
</tr>
<tr>
<td>Costelytra zealandica (White)</td>
<td>native invader</td>
<td>New Zealand</td>
</tr>
<tr>
<td>Diloboderus abderus (Sturm)</td>
<td>native invader</td>
<td>South America</td>
</tr>
<tr>
<td>Heteronychus aurator (Fabricius)</td>
<td>native invader</td>
<td>South Africa</td>
</tr>
<tr>
<td>Holotrichia spp.</td>
<td>native invader</td>
<td>South Asia</td>
</tr>
<tr>
<td>Melolontha melolontha Linnaeus</td>
<td>native invader</td>
<td>Western Europe</td>
</tr>
<tr>
<td>Odontria spp.</td>
<td>native invader</td>
<td>New Zealand</td>
</tr>
<tr>
<td>Oryctes rhinoceros (Linnaeus)</td>
<td>native invader</td>
<td>Asia/Pacific</td>
</tr>
<tr>
<td>Phylophaga spp.</td>
<td>native invader</td>
<td>Mexico, Colombia</td>
</tr>
<tr>
<td>Pyronota spp.</td>
<td>native invader</td>
<td>New Zealand</td>
</tr>
<tr>
<td>Tomarus subtropicus (Blatchley)</td>
<td>native invader</td>
<td>North America</td>
</tr>
</tbody>
</table>

1.4.3. *Costelytra zealandica* - a model species

The scarab *C. zealandica* is one of the numerous native insects to have become a major pest in New Zealand (Scott 1984, Grimont et al. 1988). Before European settlement, the habitats of this insect were the native tussock grassland and grassland margins of the bush (Kelsey 1957, Hunt 2004). Subsequently, the replacement of native forests and grasslands with plantation pastures and crops along with the introduction of over 700 exotic plants (McDowall 1994, Lee et al. 2006) has resulted in the contraction of native plant distribution ranges and the exploitation of modified habitats by native species (Yeates 1991). Both the introduction of planted exotic grasses to transform the bush and tussock into pasture and the introduction of crops and other cultivated plants have disturbed the equilibrium established between *C. zealandica* and its native environment (Yeates 1991, Hunt 2004). Following the intensification of agriculture, this endemic
New Zealand scarab has become a major pest for pasture, berry fruit, seed crops and many other cultivated plants (East & Pottinger 1984, Scott 1984, Richards et al. 1997), causing up to $89 million in lost production per year (Garnham & Barlow 1993). Interestingly and in contrast, *Costelytra brunneum* (Broun) (Coleoptera: Scarabaeidae), a close congeneric species, has never reached the status of invader and remains mostly confined to New Zealand native habitats (Given 1966, Lefort et al. 2012, 2013).

Munoz & Ackerman (2011) recognised the comparison of non-invasive and invasive congeners as a useful approach to study invasiveness in plant species. In a similar way, the comparison of these two close congeneric scarabs offers a new opportunity to investigate the mechanism(s) underlying invasiveness in native species and in phytophagous insects in general.

### 1.5. Research rationale and overall aim of the thesis

As mentioned earlier, despite the existence of numerous studies that attempt to identify key traits that may result in an insect species becoming invasive, the reasons why some species become invasive and other closely related species do not, remains unclear. Among the possible explanations for insect invasion success, the ERH (Elton 1958) is certainly the most often invoked. However, for an endemic or a native species that becomes invasive in its home range, that theory is not as tenable. This suggests that other mechanisms may have led or contribute to their invasion success.

The overall aim of the present thesis is to investigate potential alternative mechanisms and to identify novel traits that could characterise native phytophagous insect invasion success using the endemic scarab beetles *C. zealandica* and *C. brunneum* as a model system. The comparison of the relatively recent emergent pest *C. zealandica* and its non-pest congener *C. brunneum* offers a novel opportunity to explore the reasons for the development, or not, of invasiveness when presented with a novel host.

Ultimately, this research is expected to contribute to greater predictive ability around the invasive potential of phytophagous insect species in general, and to suggest as yet unexplored traits that could be incorporated into risk models and used towards protecting vulnerable managed and indigenous ecosystems.
1.6. Hypotheses and research objectives

To explore putative mechanisms and characterise novel traits associated with the development of invasive characteristics and the invasion success of native phytophagous insects, three main hypotheses have been developed. Each will be tested by addressing a series of research objectives, which will be covered by one or another chapter of this thesis.

1.6.1. Hypotheses

**Hypothesis #1.** A phytophagous insect that becomes a successful invader on a new host in its native range has higher fitness on that host compared with its ancestral host, and in contrast with a closely related non-pest species.

**Hypothesis #2.** A phytophagous insect that has higher fitness on a new host species is better able to overcome the defences of that new host compared with a closely related non-pest species.

**Hypothesis #3.** A phytophagous insect that becomes a successful invader on a new host in its native range is more tolerant of a wider range of environmental conditions, particularly with regard to temperature, in contrast to a closely related non-pest species.

1.6.2. Objectives

The objectives established to address the three hypotheses of this thesis are:

1. To develop non-invasive molecular methods to differentiate and identify the two model species used as the subject of this research (research hypotheses #1, 2 and 3).
2. To determine if and how different host plant/temperature conditions and combinations affect fitness responses of the native pest species (research hypotheses #1 and 3).
3. To determine the feeding preferences and growth responses of the two model species to native and exotic hosts (research hypothesis #1).
4. To test the ability of the two model species to overcome and/or tolerate the artificially increased defences of an exotic host plant (research hypothesis #2).
(6) To investigate the molecular expression of phosphoglucone-6-isomerase (Pgi) gene as a potential intrinsic characteristic of environmental tolerance in the native pest species (research hypothesis #3).

1.7. Thesis structure

The present thesis is organised as follows: an introduction (Chapter one), seven research chapters among which several are based on published material or submitted manuscripts, divided into three methodological chapters (Chapters 2 to 4) and four experimental chapters (Chapters 5 to 8), a discussion (Chapter 9), general references, appendices and copies of published papers. In Chapters 3 to 7, some sections were reproduced close to their published/publishable form with minor editing to avoid unnecessary repetition between chapters. These chapters start with a note regarding the status of the manuscript(s) on which the chapter is based: published or submitted. Although not published nor submitted, Chapter 8 was written as a stand-alone chapter to maintain style consistency between the research chapters of this thesis.

Chapter 1. General introduction.

Chapter 2. Methodological chapter. The aim of this short chapter is to cover the various methodological aspects relative to this thesis to avoid unnecessary repetition in the various methodological sections of the subsequent chapters. Generic sampling and rearing techniques as well as information about the insects and host plants used for the present research are described.

Chapter 3. Methodological chapter. This chapter presents how the technical challenges imposed by the use of live insect specimens and cryptic larvae were resolved via the development of new molecular techniques.

Chapter 4. Methodological chapter. This chapter presents new taxonomical and morphological findings that allow accurate distinction between the cryptic larvae of the pest C. zealndica and the non-pest C. brunneum.

Chapter 5. Experimental chapter. This chapter explores the feeding preferences and performance of the two congeneric scarabs used as the species model and aims to highlight new insights about the mechanisms that could underpin the establishment of invasive characteristics in the pest species C. zealndica.
Chapter 6. Experimental chapter. This chapter aims to establish whether the variation in the diet breadth, and eventually the invasion process, of the pest species *C. zealandica* relies on a host shift or on a host range expansion.

Chapter 7. Experimental chapter. This chapter investigates the fitness of the model scarab species in response to host defences artificially triggered and enhanced by the phytohormone jasmonic acid. The aim of this chapter is to determine whether or not the pest *C. zealandica* gained its status of biological invader through a pre-existing ability to tolerate the detrimental effects of its host’s defence chemicals.

Chapter 8. Experimental chapter. This chapter explores the thermal tolerance of the scarab species model as a factor relating to invasiveness capability. It also investigates the possibility of the metabolic enzyme-system phosphoglucose-6-isomerase (PGI) as a key system in the invasion success of these insects.

Chapter 9. General discussion and concluding remarks.
Chapter 2

General sampling and culturing methods for *Costelytra* (Scarabaeidae: Melolonthinae)

2.1. Introduction

In 1952, Given described the New Zealand endemic scarab genus *Costelytra* (Scarabaeidae: Melolonthinae), which has become best known for the pest status of one of its 11 species. The common grass grub, *C. zealandica*, quickly invaded newly developed European pastoral ecosystems in its native home range, causing substantial damage to introduced plant species and rapidly becoming one of New Zealand’s worst pests (Pottinger 1975; Richards et al. 1997). Although both the adults and the larval stage of this species are considered harmful, most plant damage is caused by 2nd and 3rd instar larvae feeding on root material (Fenemore & Perrott 1970, Chapman in Scott 1984). As a consequence, during the last decades, research has focussed on the management of these immature life-stages (e.g., Sutherland et al. 1975; Miln 1978; Glare 1992; Gatehouse et al. 2009), and most knowledge regarding the genus *Costelytra* is based on this species alone. Far less is known about *C. brunneum*, another representative species of this genus that, similarly to *C. zealandica*, occurs in both the North and South Islands of New Zealand (Given 1966), but which has never reach the status of invader.

Short reviews regarding the biology, life cycle and feeding habits of the pest species *C. zealandica* were used as a framework to develop this chapter, which presents and discusses the main methods, from field sampling to insect and plant culturing techniques, employed in this thesis.
2.2. Insect sampling and general laboratory rearing methods

2.2.1 Sample sites

*Costelytra zealandica* has a wide distribution throughout New Zealand that goes from sea level up to 1200 m (Given 1966, Scott 1984, Richards et al. 1997) and has been recorded as far as the Chatham Islands. This species seems to be absent only in the higher zones of the Alpine area (Bain 1980). The larvae of this insect are considered generalist root feeders (Given 1952) that feed on a wide variety of native and exotic plants (Table 2.1).

**Table. 2.1.** Non-exhaustive list of *Costelytra zealandica* native and exotic host-plants, regardless of the life-stage of the insect.

<table>
<thead>
<tr>
<th>Host-plant</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coprosma spp.</td>
<td>native</td>
</tr>
<tr>
<td>Corynocarpus laevigatus</td>
<td>native</td>
</tr>
<tr>
<td>Discaria tournatou</td>
<td>native</td>
</tr>
<tr>
<td>Festuca novae-zealandica</td>
<td>native</td>
</tr>
<tr>
<td>Kunzea ericoides</td>
<td>native</td>
</tr>
<tr>
<td>Poa caespitosa</td>
<td>native</td>
</tr>
<tr>
<td>Poa colensoi</td>
<td>native</td>
</tr>
<tr>
<td>Sophora spp.</td>
<td>native</td>
</tr>
<tr>
<td>Actinidia deliciosa</td>
<td>exotic</td>
</tr>
<tr>
<td>Berberis spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Brassica spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Citrus spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Crataegus spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Cydonia oblonga</td>
<td>exotic</td>
</tr>
<tr>
<td>Daucus carota</td>
<td>exotic</td>
</tr>
<tr>
<td>Fragaria spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Lomatium perenne</td>
<td>exotic</td>
</tr>
<tr>
<td>Lonicera spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Lycium spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Malus domestica</td>
<td>exotic</td>
</tr>
<tr>
<td>Pluus spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Platanus spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Prunus spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Pyrus spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Ribes uva-crispa</td>
<td>exotic</td>
</tr>
<tr>
<td>Rosa spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Rubus spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Salix spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Trifolium spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Ulex spp.</td>
<td>exotic</td>
</tr>
<tr>
<td>Vitis spp.</td>
<td>exotic</td>
</tr>
</tbody>
</table>
Consequently, numerous sample sites were available for this species, and their selection was essentially based on (1) the nature of the plants encountered on the site (i.e. exotic pastoral plants versus native grassland-type plants), (2) the proximity of the site from the laboratory to minimise transportation time and associated stress on the insect, in order to avoid adverse effects on the results of subsequent fitness-related experiment, (3) the degree of infestation of the site by the species (i.e. highly infested sites where larvae occurred in high densities were preferred to optimise sampling effort), and (4) the history of the site, where the likely occurrence of pathogens capable of affecting this species were reported as being low (personal communication, Richard Townsend, AgResearch NZ). Based on these characteristics, four sites (i.e. A, B, C and E) were chosen as *C. zealandica* larvae sample sites (Figure 2.1) and the characteristics of each of them are summarised in Table 2.2.

**Table 2.2.** General description and location for *Costelytra zealandica* and *C. brunneum* sample sites, the year(s) they have been exploited and their use for the experiments in this thesis.

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Coordinates</th>
<th>Site description</th>
<th>Year(s)</th>
<th>Chapter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Lincoln (NZ, South Island)</td>
<td>43°64'04''S 172°47'82''E</td>
<td>mixed exotic ryegrass / clover garden</td>
<td>first year</td>
<td>3 and 5</td>
</tr>
<tr>
<td>B</td>
<td>Hororata (NZ, South Island)</td>
<td>43°32'17''S 171°57'16''E</td>
<td>mixed exotic ryegrass / clover dairy pasture</td>
<td>first and second years</td>
<td>3, 4, 5, 6, 7 and 8</td>
</tr>
<tr>
<td>C</td>
<td>Cass (NZ, South Island)</td>
<td>43°02'10''S 171°45'40''E</td>
<td>native tussock grassland</td>
<td>first and second years</td>
<td>3, 5, 6 and 7</td>
</tr>
<tr>
<td>D</td>
<td>Castle Hill (NZ, South Island)</td>
<td>43°12'20''S 171°42'16''E</td>
<td>native tussock grassland close to the margin of beech (Nothofagus) forest</td>
<td>first and second years</td>
<td>3, 4, 5, 7 and 8</td>
</tr>
<tr>
<td>E</td>
<td>Te Awamutu (NZ, North Island)</td>
<td>38°09'95''S 175°35'07''E</td>
<td>mixed exotic ryegrass / clover dairy pasture</td>
<td>second year</td>
<td>8</td>
</tr>
</tbody>
</table>

![Figure 2.1. Location map for *Costelytra zealandica* and *C. brunneum* sample sites.](image)
In contrast to its congener *C. zealandica*, very little is known about the exact geographical distribution of *C. brunneum*. Given (1996) roughly described its range as ‘wide but discontinuous’ around New Zealand. Sample site selection for this species was consequently not as straightforward as for *C. zealandica*. Site identification was therefore based on information gathered following the examination of adult specimens from insect museum collections (i.e. Ministry of Primary Industry Investigation and Diagnostic Centre of Christchurch and Entomological Museum of Lincoln University) as well as personal communication (Richard Townsend, AgResearch NZ). Based on the above, two collections sites (i.e. C and D), located in the South Island of New Zealand, were selected to collect the larvae of *C. brunneum* (Figure 2.1 and Table 2.2).

Because samples were collected in two consecutive years (i.e. first or second year of the PhD research project) and the number of individual collected during each sampling sessions varied, not all species / populations could be used in all experiments of this thesis (Table 2.2). Therefore, the collection sites used in the subsequent chapters vary based on the year the experiments were performed (Table 2.2).

### 2.2.2 Larval sampling

*Costelytra zealandica* is considered as an univoltine organism (Atkinson & Slay 1994), although it is not uncommon to come across individuals that follow a two-year life cycle in the highest and coldest environments of the southern locations of New Zealand, such as Otago and Southland (Stewart 1972, Kain 1975). With the exception of the adult beetle, all life stages of *C. zealandica* are entirely subterranean in habit (Kain 1975).

*Figure 2.2. Costelytra zealandica univoltine life-cycle*
Adult beetles of *C. zealandica* emerge from their pupal case and move to the soil surface between mid-October and mid-December (East and Kain 1981) (Figure 2.2). Females are in general quickly mated as soon as they reach the surface of the soil (East and Kain 1981). Subsequently, the females usually oviposit close to the spot from which they have emerged (Fenemore & Perrott 1970, Farrell 1972), which often results in a quite localised area of infestation (Fenemore, 1984). According to East and Kain (1981), these clusters can comprise up to 50 eggs that are laid between 8 - 25 cm depth in the soil, although Wright (1989) suggests that the clusters probably contain no more than 30 eggs and might be laid between 2.5 – 15 cm in depth only. By the end of December, most eggs hatch into first instar larvae (Figure 2.2). This stage predominates during January, although the second instar stage can be found from early January to March with a maximum population usually reached in February (East & Kain 1981, Wright 1989) (Figure 2.2). The third instar larvae can be found in soil from February to September (Figure 2.2), although the larvae usually cease to feed and move deeper into the soil to construct their pupal cell from late June (East & Kain 1981, Wright 1989). The pupal stage lasts for several months, from late August until the emergence of the adult beetle (East & Kain 1981) (Figure 2.2). To date, *C. brunneum* has been poorly studied and the only indications available about its life cycle are that it may follow a similar seasonal cycle as its congener *C. zealandica*, with the exception that adults may emerge a few weeks earlier (Given 1952).

The most damaging stage of *C. zealandica* is often considered the third instar (Fenemore 1984), because of its extensive duration and also because, in the first half of this final larval stage, larval feeding increases significantly to accumulate sufficient reserves for pupation (pre-pupal stage). The third instar larvae were therefore selected as the experimental stage for both species, in most experiments presented in this thesis. Sampling campaigns to collect third instar larvae were consequently organised in February over two seasons (Table 2.1).

![Figure 2.3. First 20 cm of the sample site soil turned over with several specimens of second and third instar larvae of *Costelytra* sp.](image-url)
To sample larvae, the first 20 cm of soil was dug out using a spade and larvae carefully collected by hand (Figure 2.3). Because larval combat may occur at high densities when larvae come into contact with each other (East et al. 1981, Van Den Bosch et al. 1995), larvae where placed in large compartmented boxes (Figure 2.4), with no more than 15 larvae per compartment for transportation to the laboratory. Sample sizes for each experiment performed in this thesis were determined in consultation with a statistician.

Figure 2.4. Compartmented boxes (40 x 25 x 55 cm) used to transport collected larvae, from the field to the laboratory, to avoid larval-combat and associated mortality.

2.2.3 Insect rearing

2.2.3.1 Amber disease preliminary test

*Costelytra zealandica* has numerous natural enemies including bacteria, fungi and protozoan species (Jackson 1990, Glare 1992). The most common disease affecting *C. zealandica* appears to be amber disease (Jackson 1990). This chronic infection of the larval gut is caused by bacteria belonging to the genus *Serratia* (Proteobacteria: Enterobacteriaceae) (Grimont et al. 1988, Jackson et al. 2001). When affected, the larva stops feeding within 48 hours, its gut clears completely of its content and 72 hours after the initial infection the larva will show a characteristic amber coloration (Jackson et al. 1993). This chronic infection is usually accompanied by a gradual weight loss and generally leads to the death of the larva in four to six weeks (Grimont et al. 1988, Jackson et al. 2001), although death may occur later depending on abiotic factors such as temperature (Jackson et al. 2001).
Amber disease is suspected to be extremely host-specific (Hurst and Jackson 2002), but as a matter of equity and as a preventive measure, all the collected larvae, of both *C. zealandica* and *C. brunneum* were tested for this disease prior to experiments. The specimens were initially placed individually in ice tray pack compartments with a piece of carrot at 15°C ambient temperature for four days according to Jackson et al. (1993) protocol (Figure 2.5). Only few unhealthy larvae (<1%) were identified as they did not feed and were therefore discarded.

![Figure 2.5. Amber disease-free test, where Costelytra spp. larvae were placed individually in ice tray pack compartments with a piece of carrot at 15°C ambient temperature for four days.](image)

### 2.2.3.2 General rearing-soil and temperature conditions

Although scarab larvae generally prefer humid and even occasionally waterlogged soils (Galbreath 1976), excessive soil moisture conditions may cause high mortality in *C. zealandica* (Kain 1975). Soil moisture seems to also impact on *C. zealandica* larvae vertical distribution and Farrell (1972) observed that under summer drought conditions larvae were found deeper in the soil, presumably to damper areas, because of an inability to feed close to the soil surface.

*Costelytra zealandica* is known to have a large range of ecological tolerances (Given 1952), which includes the type of soil in which it develops. *Costelytra zealandica* preferences with respect to soil types has been investigated many times (Kain 1971, Farrell 1972, Van Den Bosch et al. 1995) and is dependent upon the host plant(s) encountered on the site.

*Costelytra* larvae are not obligate root feeders but in certain situations have the ability to grow on humus or organic material (Sutherland 1971). Hence, the pasture soil collected to rear the larvae of both species of *Costelytra* was carefully prepared, by first being passed through a 2 mm sieve to remove any large residual plant material and then treated by gamma-irradiation (Schering-
Plough Animal Health, Wellington, NZ) to eliminate any residual organic matter and potential pathogens.

In the 1970s, numerous in-situ experiments, designed to study *C. zealandica*, used a room temperature comprised between 16 and 20°C (e.g. Henzell et al. 1970, Wightman 1972, Wightman 1974, Kain & Atkinson 1977). However more recent experiments have revealed that rearing *C. zealandica* larvae at 15°C was highly successful (Richard Townsend & Travis Glare personal communication, AgResearch NZ).

As a standard, each larva of both species was kept individually in a 35 ml plastic container with 50 g of irradiated soil (Figure 2.6) and kept in incubator at controlled temperature of 15°C, which was monitored two-hourly by a USB data-logger device. Ambient moisture in the incubators was set up at 35% humidity, although soil humidity was probably highly dependent upon the ambient humidity of the tube itself. Hence, in order to ensure and maintain sufficient moisture, cotton wool soaked with water was placed in each container and three holes were pierced on their lid to allow sufficient airflow.

![Figure 2.6. Standard rearing condition for *Costelytra* larvae. A 35 ml plastic container filled up with 50 g of irradiated soil, with a cotton wool soaked with water to maintain humidity and a lid pierced with three holes to allow sufficient airflow.](image)

### 2.2.4 Plant cultures

As mentioned earlier, and pointed out by Given (1952) when he described the genus *Costelytra*, the larvae of *C. zealandica* have a wide range of ‘ecological tolerance’ particularly regarding their food consumption. Throughout its entire life cycle, this species can feed on an extensive range of exotic host (Table 2.1). Very little is known about the feeding habits of *C. bruneum*. As for its congeneres, the larvae of this species are root feeders, but are likely to display a much more
limited tolerance for food than C. zealandica. Adults of C. brunneum have been mostly found and reported to feed on native host plants such as the ones found in local native grasslands (e.g. different varieties of tussock and fescue) and sometimes on the margins of the surrounding bush (R. Townsend, personal communication, AgResearch NZ). There are few reports of adults occurring within proximity of exotic pastures and in private gardens (Barbara Barratt, personal communication, AgResearch NZ), but the somewhat mimetic aspect of C. zealandica and C. brunneum could have caused misidentification, particularly of the less common non-pest species, and thus reducing reports of its occurrence.

Based on the current knowledge regarding C. zealandica plant consumption, and the few reports of C. brunneum feeding habits, four host plants were selected to perform the various experiments presented in this thesis. The exotic host-plants, Trifolium repens (white clover) and Lolium perenne (ryegrass) were grown from seeds in a nursery in 200 ml of potting mix comprising 60% peat and 40% sterilized pumice stones. Poa cita (silver tussock) and Festuca novae-zealandiae (fescue tussock) were chosen as native hosts. Young plants of these two species were purchased from nursery, Trees for Canterbury, (Christchurch, NZ). Each plant was carefully transferred from its original pot to a 200 ml pot, filled up with potting mix comprising 60% peat and 40% sterilized pumice stones, and grown for 2 months.

2.3. Conclusion

The lack of published data regarding the non-pest species C. brunneum first appeared challenging. From site selection to laboratory general rearing conditions, many impediments were overcome by the use and the help of the knowledge of local people and scientists that have at some point come across or researched this species. Furthermore, it appeared that general culturing conditions developed to rear the larvae of C. zealandica were also appropriate for C. brunneum.

To summarise, key decisions and culturing techniques employed in this thesis were:

1. A selection of five sites to collect the larvae of the species model.
2. Samples collected in February and use of compartmented boxes to transport the larvae to avoid larval combat.
3. A preliminary amber disease-free test prior to each experiment.
4. The use of individual containers containing irradiated (i.e. sterile) soil and kept 15°C as a standard larvae rearing condition.
5. A selection of four hosts, comprising two exotic and two native plants to perform the various experiments presented in this thesis.
Chapter 3

Non-invasive molecular methods to identify cryptic and live scarab larvae

Results of this chapter published as


Abstract

Despite the negative impact that many scarab larvae have on agro-ecosystems, very little attention has been paid to their taxonomy. Their often extremely similar morphological characteristics have probably contributed to this impediment, which has also meant that they are very difficult to identify in the field. Molecular methods can overcome this challenge and are particularly useful for the identification of larvae to enable management of pest species occurring sympatrically with non-pest species. However, the invasive collection of DNA samples for such molecular methods is not compatible with subsequent behavioural, developmental or fitness studies. Two noninvasive DNA sampling and DNA analysis methods suitable for the identification of larvae from closely related scarab species were developed here. Using the frass and larval exuviae as sources of DNA, field-collected larvae of this thesis model species Costelytra zealandica (White) and Costelytra brunneum (Broun) (Scarabaeidae: Melolonthinae) were identified by multiplex PCR based on the difference in size of the resulting PCR products. This study also showed that small quantities of frass can be used reliably even seven days after excretion. This stability of the DNA is of major importance in ecological studies where timeframes rarely allow daily monitoring. The approach developed in this chapter is readily transferable to the study of any holometabolous insect species for which morphological identification of larval stages is difficult.

3.1. Introduction

Many larvae of root-feeding insect species are regarded as pests (Blossey and Hunt-Joshi 2003, Dittrich-Schröder et al. 2009). Among these, scarabs are generalist root feeders (Cowles et al. 2005) of which several species affect a wide variety of economically important pasture, agricultural and horticultural plants (McPeack 2006, Romero-López et al. 2010). Most often it is
the larvae of these species that cause the damage and consequently research to improve their management tends to focus mainly on that life stage. However, surprisingly, and as highlighted by Dittrich-Schröder et al. (2009), very little attention has been paid to the taxonomy of scarab larvae and so the ability to identify them morphologically to species level is very difficult. This is compounded by the larvae of closely related species looking very similar in size, colour and shape (Bain 1980), and taxonomic keys to distinguish them are underdeveloped and generally rely on the use of minute morphological characteristics often only accessible by dissection. Therefore the likelihood of misidentification of field-collected larvae, particularly where two or more species co-occur (e.g. Miller et al. 1999) is high. Accurate species identification of scarab larvae is crucial to prioritise the correct species-specific treatments for pest management, especially when only one of the species present is regarded as a threat and when laboratory studies on live specimens are required.

One way of identifying field-collected larvae of scarabs is to conduct molecular analyses that can link larval genetic profiles to those of identified adult specimens. In the past this approach has been successfully achieved using the convenience of RFLP analysis for many scarab (e.g. Miller et al. 1999) and other species (e.g. Armstrong et al. 1997). More recently an improved approach has been to use DNA barcoding (Hebert et al. 2003) for various invertebrates (Armstrong & Ball 2005, Waringer et al. 2008, Zhang et al. 2008, Zhang & Weirauch 2011) including scarabs (Miller et al. 2005, Dittrich-Schoder et al. 2009). However, this usually requires invasive tissue sampling for DNA extraction purposes, which is clearly not compatible with subsequent behavioural, developmental or fitness studies requiring physiologically unaffected live specimens. The alternative is to use non-invasive DNA sampling methods such as those developed to comply with animal welfare (e.g., Beja-Pereira et al. 2009) or for the conservation of endangered species (e.g., Gregory & Rinderer 2004, Beja-Pereira et al. 2009, Monroe 2010). The above methods however were mostly developed for rather large animals, with for example the use of shed hair and feathers, or saliva (Taberlet & Luikart 1999) and are generally not appropriate for much smaller animals such as insects. As a consequence, non-invasive DNA sampling methods for the identification of living insects is not common and has been rarely attempted (Feinstein 2004).

In this chapter, several non-invasive DNA sampling and DNA analyses methods suitable for the identification of closely related scarabs are described. These methods were tested using the model species of this thesis, *C. zealandica* and *C. brunneum*, for which populations can occur in sympatry. Accurate distinction between these two species is important because *C. zealandica* is a significant pest in pastures for which early detection at the larval stage is crucial for its control. Unfortunately, taxonomic keys are only available for the adult life stage of this genus (Given 1952, 1966). Therefore, distinguishing the presence of *C. zealandica* from that of the non-pest *C. brunneum*, which does not reach damaging population densities, is difficult, and leads to the potentially unnecessary implementation of expensive management strategies. Furthermore,
reliable non-invasive identifications techniques to distinguish the two species were required to pursue the various experiments presented in this thesis.

3.2. Material and methods

3.2.1. Insect sampling

All adult beetles were either sampled by light trapping or by opportunist catching. *Costelytra brunneum* were collected from Cass (n=3) and *C. zealandica* from Christchurch (n=2), Kaikoura (n=2) and Picton (n=1). Their morphological identification was based on Given's keys (1952, 1966).

Larvae were collected following the methodology described Chapter 2, from the sample sites A (n=10), B (n=15), C (n=89) and D (n=28) (Section 2.2.1). All larvae were identified as *Costelytra* based on Given’s key of New Zealand Melolonthinae (1952) and in accordance with external characters such as body length and relative head capsule size (Given 1952).

3.2.2. Species-specific primer design and diagnostic assay by multiplex PCR

DNA extractions were performed with the Zymo Research Insect/Tissue DNA Kit-5 using one leg from each of three morphologically identified adults of *C. brunneum* and five of *C. zealandica*. Universal primers LCO1490 and HCO2198 (Folmer et al. 1994) were used to amplify a 658 bp fragment of the cytochrome oxydase 1 gene (COI) by Polymerase Chain Reaction (PCR), using Expand High Fidelity Enzyme Mix (Roche Applied Science) and reaction conditions according to the manufacturers protocol. The PCR products were prepared for DNA sequencing with BigDye terminator kit according to the manufacturers protocol (Applied Biosystems, Foster City, CA) plus the same primers as used for the PCR. The sequence products were analysed on an ABI 3130xl (Applied Biosystems, Foster City, CA) DNA sequencer. Forward and reverse sequences from the same specimen were aligned using MEGA 4 (Tamura et al. 2007) (Genbank accession numbers JN793483-JN793487 and JN793498-JN793500). BLASTn searches (Karlin & Altschul 1990) of the GenBank database were performed to confirm that the obtained sequences most closely matched those of other Melolonthinae species. Multiple sequence alignments were performed and a consensus sequence was constructed for each of the two species studied using MEGA 4 (Tamura et al. 2007) (Figure 3.1).
**Figure 3.1.** Consensus sequences alignment of the mitochondrial region cytochrome oxydase 1 gene (COI) for adult specimens of *Costelytra zealandica* (n = 5) and *C. brunneum* (n = 3). Intraspecific variabilities are highlighted by degenerate positions in clear boxes. Grey boxes indicate the location of the species-specific primers designed and used for this study (LC01490 as forward primer and COI_Czeal_FolB and COI_Cbrun_FolB as reverse primers).

Based on the consensus COI sequences, species-specific reverse primers were designed, COI_Czeal_FolB (5'-GTGATAGCTCCTGCTAATACAGGTAAA-3') for *C. zealandica* and COI_Cbrun_FolB (5'-ACCGGCTCCGGTTTCGAT-3') for *C. brunneum*, both to be used with the forward primer LC01490 (5'-GGTCAACAAATCATAAGATTTG-3') (Folmer et al. 1994). These primers pairs were designed to amplify fragments of distinct sizes, so that the respective amplicons (Figure 3.1) could be distinguished in a single multiplex PCR. Multiplex PCRs were performed using the GoTaq Green Master Mix (Promega). Each reaction contained 1x GoTaq®
Green Master Mix, 0.3 μM of each primer, 2μl of DNA template and made up to 20 μl with nuclease-free water. PCR reactions were prepared under a sterilised UV hood, filter tips were used to avoid cross-contamination and negative controls (i.e., water + PCR mix) were included in each PCR.

The species-specific primers and multiplex assay were subsequently tested on DNA extracted (as above for the adult specimens) from single legs of 122 unidentified Costelytra larvae (from the sample sites A, C and D). DNA of the morphologically identified adult specimens were used as positive controls. Size of the PCR products, as an indicator of species identification, was measured after electrophoresis on 2% agarose gels. A total of 20 PCR products (10 of each amplicon size) were sequenced (Genbank accession numbers JN793488-JN793497 and JN793501-JN793510) following the method mentioned earlier and compared to those of identified adults.

3.2.3. Additional diagnostic by restriction enzyme analysis

An additional molecular tool based on restriction enzyme digestion of the full Folmer region of the COI gene was also developed. This used the universal primer pair LCO1490 and HCO2198 (Folmer et al. 1994) in the event that the specific primers designed gave ambiguous results. The consensus COI sequences for C. zealandica and C. brunneum revealed likely species-specific restriction enzyme sites. Of these, digestion using Dra I (TTT ↓A°AA) would yield a restriction pattern of two fragments of 268 bp plus 442 bp for C. zealandica and 622 bp plus 88 pb for C. brunneum.

3.2.4. Non-invasive DNA sampling methods

3.2.4.1. Frass experiment

Ten unidentified third instar larvae of Costelytra (from collection B) were used to provide frass (Figure 3.2a) over the experimental period. Larvae were placed individually in a compartment of a 12 well ice-cube tray with a 1 cm³ piece of carrot as per standard culturing conditions (Villalobos et al. 1997). Over a 48 hour period, all frass produced were collected, individually weighed and stored in 1ml plastic vials. These were kept at room temperature (~20°C) for a range of experimental time periods. The average weight of a frass pellet produced by a larva was 0.199 ± 0.048 mg. To determine the minimum amount of frass required to successfully identify each larva, DNA extractions were performed on samples made of various frass quantities (12, 8, 4, 2, 1 and ½ frass pellet(s)). For each of these quantities, five larvae were selected and their frass analysed at day one, three and seven to check the stability of DNA over time. DNA extraction and multiplex
PCR were conducted as above, with 0.2 μg/μl of purified bovine serum albumin (BSA) added to each PCR reaction.

### 3.2.4.2. Exuviae experiment

Ten unidentified second instar larvae (from sample sites B and D) were placed individually in a compartment of a 12 well ice-cube tray with soil from the sample site, and a 1 cm$^3$ piece of carrot. Larvae were checked daily and exuviae (Figure 3.2b) collected as soon as they were physically detached from the larvae. Exuviae were kept at -20°C in 1 ml individual plastic vials. DNA extraction was performed on the whole exuviae chopped into small pieces. Protocols for DNA extraction, amplification and analysis were the same as for the frass experiment.

![Figure 3.2. Non-invasive scarab DNA samples, with (a) single adult Costelytra spp. frass and (b) Costelytra spp. second instar larvae exuviae. Scale 0.5 mm.](image)

### 3.3. Results

#### 3.3.1. Specific primers diagnostic assay by multiplex PCR

Single PCR products, were produced using the combined species-specific primer pairs with DNA from identified adult specimens of the two species and were clearly distinguishable between the two species by ~240 bp (Figure 3.3). This same multiplex assay successfully amplified DNA from each of the 122 larvae. Of these, a total of 96 specimens displayed the 546 bp COI fragment of *C. zealandica*, while 26 specimens displayed the 304 bp COI fragment of *C. brunneum* (Figure 3.3). Ten examples of each were sequenced and confirmed that these were the targeted amplicons (Genbank accession numbers JN793488-JN793497 and JN793501-JN793510). Hence, the additional diagnostic confirmation by restriction enzyme analysis appeared unnecessary.
3.3.2. Non-invasive DNA sampling methods

3.3.2.1. Frass experiment

Considering all treatments combined, more than 88% of the frass DNA amplifications successfully produced a single product from the multiplex PCR (Figure 3.4). Results are summarised in Table 3.1.

Figure 3.3. Two per cent agarose gel depicting PCR amplification of mitochondrial DNA extracted from the leg and exuviae of *Costelytra zealandica* and *C. brunneum* larvae together with negative and positives controls. Three microlitres of each PCR product were loaded on the gel.

Figure 3.4. Two per cent agarose gel depicting PCR amplification of mitochondrial DNA extracted from the variables quantities of *Costelytra zealandica* frass (n = 0.5–12) together with negative and positives controls. All the frass were between 3 and 4 days old. Three microlitres of each PCR product were loaded on the gel.
Unsuccessful amplifications only occurred for low quantities of frass (i.e. 1 and $\frac{1}{2}$ frass pellet, which correspond to quantities less than $\sim 0.2$ mg of excrement) and particularly after the longest period of 7-8 days of storage (Table 3.1).

Table 3.1. Number of positive DNA amplifications ($n = 5$ for each treatment) for different frass quantities after 1–2, 3–4 and 7–8 days of storage at room temperature

<table>
<thead>
<tr>
<th>Number of frass</th>
<th>1–2 days old</th>
<th>3–4 days old</th>
<th>7–8 days old</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
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<td>4</td>
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<tr>
<td>2</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3.2.2. Exuviae experiment

The DNA of all 10 exuviae used in this assay were successfully extracted and amplified. PCR products from seven of those were equivalent to the 546 bp fragment (Figure 3.3), indicating that they were from *C. zealandica*. PCR products from the remaining three exuviae were equivalent to the 304 bp fragment (Figure 3.3) and were consequently considered to be *C. brunneum*.

3.4. Discussion

A convenient two-pronged non-invasive approach has been developed here to distinguish live larvae of two sympatrically occurring scarab species. Firstly, a multiplex PCR analysis, utilising a species-specific modification of the generic DNA barcode region (Hebert et al. 2003) was highly successful in differentiating *C. zealandica* and *C. brunneum* larvae. Unlike DNA barcoding, which requires additional post PCR sequencing for each individual, the assay was well suited to the effective screening of large numbers of samples. Incorporating three primers in a single multiplex reaction instead of two separate species-specific reactions also made the procedure much simpler and more efficient in both design and deployment. Secondly, non-invasive DNA sampling approaches were successfully applied enabling accurate species differentiation of live specimens.

Because scarabs have complex and long life cycles (Ritcher 1957), their culturing under laboratory conditions can be very challenging (Dittrich-Schröder et al. 2009) and is often
disregarded in favor of extensive field collections of larvae. This requires prior non-harmful and non-disruptive methods of identification to enable subsequent analyses of ecological, behavioural and fitness data, without undermining the validity of the results obtained. In such a context, combining the above rapid diagnostic PCR method with the non-invasive approach to DNA sampling is key.

Non-invasive DNA sampling methods for insects are rare and often limited to specific circumstances. For example, the whole specimen soaking in extraction buffer, specifically developed for the conservation of precious museum type specimens (Gilbert et al. 2007) is not compatible with live specimens. Another method proposed by Katoh et al. (2008), involving using the nucleotide-acid binding property of silica particles to retrieve DNA from the reflex bleeding of ladybeetles and leaf beetle also cannot be applied to scarab larvae.

Although excrements are increasingly used as source of DNA in molecular and ecological studies (Zhang et al. 2006), such an approach has rarely been applied in genetic studies that focus on arthropods. Reasons for that include (i) the presence of only small quantities of DNA (Piggott & Taylor 2003, Zhang et al. 2006), compounded by the use of small specimens, (ii) the unwanted cross-amplification of DNA (Fumanal et al. 2005) from endogenous and exogenous sources and (iii) the possible presence of highly interfering substances such as PCR inhibitors (Kohn et al. 1995, Monteiro et al. 1997).

The results of this study showed that, in the case of scarab species, as little as half a frass pellet can be sufficient to successfully amplify the target DNA. In addition, DNA amplification was successful even up to four days following excretion, and, in the majority of cases, up to eight days, although a higher quantity of frass (>1 pellet) was required. This latter discovery is extremely valuable, as timeframes in ecological studies rarely allow for daily monitoring. Undoubtedly, the time limit over which such sources of DNA can be used will be dependent on the ambient temperature and moisture conditions that determine the rate of degradation of the DNA (Murphy et al. 2007). However this can easily be countered by controlled and monitored laboratory conditions and experimental design where relatively cold and dry conditions are known to be more favorable for the preservation of DNA samples (Piggott & Taylor 2003). Moisture was probably a very important parameter for the quality of the retrieved fecal DNA and the success of its amplification in the present study, as scarab larval frass appeared as being consistently dry (pers. obs.).

An alternative method for non-invasive sampling of immature life stages is the use of exuviae shed after each instar moult. Insect exuviae are essentially composed of chitin and chitosan (Zhang et al. 2000) with no nucleated cells that contain DNA. Consequently, non-molecular methods, such as the cuticular hydrocarbon composition of exuviae, have been used to identify insect larvae (e.g., Ye et al. 2007). However, some living cells are often shed during the moulting
process (Bertholf 1925) such that the low quantities of DNA can then be amplified by PCR. This constitutes an easier and cheaper alternative to cuticular hydrocarbon analyses. Several studies have successfully used exuviae as source of DNA. For example, Gregory and Rinderer (2004) obtained similar PCR results from DNA extracted from the exuviae of honey bee (Hymenoptera) larvae as from the tarsi, showing that the content of DNA in bee exuviae was enough to perform successful DNA amplification. The same year, Feinstein (2004) confirmed the utility of caterpillar (Lepidoptera) exuviae as potential source of DNA. The current study expanded these findings to coleoptera larvae. Conversely, an example of unsuccessful use of shed exuviae as source of DNA, was reported by Monroe et al. (2010) from dragonfly larvae. However these exuviae were collected directly from water, which was likely to have resulted in degradation of any residual DNA. Therefore the environmental conditions under which the exuviae are recovered are of prime importance to ensure high quality DNA for molecular analyses. This was not an issue with the species model of the present study. However, in scarabs, one of the main factors potentially restricting the use of this method is that some larvae eat their own exuviae after shedding (pers. obs.). Therefore, recovering the exuviae before they have been ingested is likely to require considerable vigilance.

3.5. Conclusion and recommendations

The methods developed and described in this study significantly improve the feasibility of using accurately identified scarab larvae in ecological and/or behavioural studies. The approach is very generic and therefore should be readily transferable to other holometabolous insect species where morphological identification of larval stages is difficult.

Requirements for successful application of the methods are:

1. Minimising the number of species-specific primers in a multiplex PCR is likely to be more robust than compromising on optimal PCR conditions when a large number of primers are involved.

2. Primers that target short fragments of DNA are more likely to amplify using degraded DNA (Idaghdour et al. 2003), such as that which might be expected from exuviae and frass.

3. Where possible, frass should be used rather than exuviae because it is produced daily and not eaten by the larvae.

4. For the species used here, a minimum of half a frass pellet (i.e. ~0.1 mg) is required for analysis after 3-4 days after excretion, or alternatively a minimum of two frass pellets (i.e. ~0.4 mg) for analysis after 7-8 days. These parameters
need to be considered on an individual study basis with respect to the species tested, ambient humidity and temperature conditions.
Combining molecular and morphological approaches to differentiate *Costelytra zealandica* from *C. brunneum* at larval stage

**Results of this chapter published as**


**Abstract**

The frequently strong morphological similarities that exist between the larvae of congeneric scarab beetles are likely to lead to misidentification of field-collected specimens of sympatric species. This is the case for the New Zealand endemic pasture pest *Costelytra zealandica* (White) (Scarabaeidae: Melolonthinae) and the closely related non-pest species *C. brunneum* (Broun), where a taxonomic key is only available for *C. zealandica* and does not provide any information that allows the larvae of the two species to be differentiated. Mistaken identification and sampling of such fundamentally different organisms during ecological and / or behavioural studies could lead to invalid interpretation and misinformed decisions especially for the establishment of pest control programmes. Molecular-based species identification is well recognised as an effective way to identify cryptic species using barcoding regions of the genome. In this chapter, this genetic approach was coupled with traditional scarab taxonomy (i.e., morphology of the raster, mandibles, labrum and epipharynx) to detect morphological characteristics that allow for a rapid and accurate differentiation between the final instar of *C. zealandica* and *C. brunneum* larvae. It was found that as few as three characters allowed an accurate identification and that the morphology of the septula of the raster itself was enough to assist with a preliminary differentiation of the two species in the field.

**4.1. Introduction**

Although both the adults and the larval stage of the pest species, *C. zealandica*, are considered harmful, most plant damage is caused by second and third instar larvae feeding on plant root...
material (Chapman in Scott 1984). Consequentially, research has tended to focus on the management of these two immature life-stages (e.g., Sutherland et al. 1975; Miln 1978; Glare 1994; Gatehouse et al. 2009), and as discussed in Chapter 3, far less attention has been paid to the basic taxonomy and ecology of the larvae, as is the case for many other scarab pest species (Dittrich-Schröder et al. 2009 Sipek & Ahrens 2011).

Morphological similarities that exist between the larvae of congeneric sympatric species are likely to lead to misidentification of field-collected specimens (e.g., Miller et al. 1999; Lefort et al. 2012). For instance, *C. brunneum* displays a patchy distribution (Given 1966; Richards 1997) that overlaps with that of the pest species *C. zealandica* in several geographic regions of New Zealand (Given 1966), causing identification difficulties. Incorrect identification of field-collected larvae can lead to invalid interpretations of data if those are used for ecological and/or behavioural studies. This is a particular problem that can be greatly compounded when the organisms have different life-histories and/or behaviour, such as a pest and a non-pest species. It is, therefore, important to develop reliable methods for both accurate and rapid differentiation of the larvae of *C. zealandica* and *C. brunneum* that have been selected as model species for this thesis.

One of the difficulties faced, when the genus *Costelytra* was described, was obtaining larvae reliably associated with corresponding known adults (Hoy & Given 1952). One reason for that is the challenge of breeding and rearing Melolonthinae species collected from mild to high altitudinal grasslands such as the ones found in the New Zealand Alps (Hoy & Given 1952). Undoubtedly, such an impediment also explains the scant attention paid to the taxonomy of numerous scarab larvae (Dittrich-Schoder et al. 2009; MacQuillan 1985; Sipek & Ahrens 2011), including those of *C. brunneum* which mainly occur at these altitudinal ranges in the South Island of New Zealand (Given 1966).

Molecular-based species identification is now recognised as a useful method for identifying specimens through comparison of DNA sequences from specific regions of the genome that are used as barcodes (Hebert et al. 2003). Such techniques have already been successfully used to link larval and adult specimens of various insects (e.g., Waringer et al. 2008; Zhang et al. 2008; Zhang & Weirauch 2011) including scarab beetles (e.g., Miller et al. 1999; Miller et al. 2005; Dittrich-Schoder et al. 2009; Sipek & Ahrens 2011) and have been developed for the model species of this thesis in Chapter 3. This work (Lefort et al. 2012) links the adults of the two *Costelytra* species to their respective larvae, which for many years were considered to be virtually impossible to tell apart (Richard Townsend, personal communication, AgResearch NZ). For practical field or laboratory-based purposes, however, a method based on traditional morphological taxonomy would clearly be advantageous.
This chapter aims to identify reliable morphological characters for the differentiation of mature larvae of *C. zealandica* and *C. brunneum* using DNA barcoding sequences of the mitochondrial cytochrome oxidase subunit 1 (COI) gene developed in Chapter 3.

### 4.2. Material and methods

#### 4.2.1. Insect sampling

Twenty-four third instar larvae were collected following the methodology described in Chapter 2. These were collected from the sample sites B and D (Section 2.2.1). Voucher specimens from the present study were deposited in the Lincoln University Entomology Research Museum collection.

#### 4.2.2. Molecular identification of the larvae

Larvae were killed by immersion for two minutes in boiling water, preserved in 70% ethanol and kept at -20°C for better DNA preservation (Schauff 1986). One leg of each specimen was carefully removed using a scalpel blade, and chopped into small pieces for DNA extraction. Extractions were performed using the Zymo Research Insect/Tissue DNA Kit-5®, following the manufacturer's protocol. The PCR reactions and conditions were performed as described in Chapter 3 (see Section 3.2.2.) using the forward primer LCO1490 (Folmer et al. 1994) and the species-specific reverse primers COI_Czeal_ and COI_Cbrun_FolB designed. The relative sizes of the amplicons obtained (i.e., 546-bp COI fragment for *C. zealandica* and 304-bp COI fragment for *C. brunneum*) were used as indicators of species identification.

#### 4.2.3. Specimen preparation and taxonomic assessment

The head capsule of four genetically identified specimens of each species was separated from the thorax using a scalpel. Head capsules were then cleaned by immersion in a 10% potassium hydroxide digestion buffer (Lawrence et al. 1993) overnight, which allowed the removal of intact ventral mouthparts as described in Dittrich-Schröder et al. (2009). The mouthparts and head capsule were kept in 70% ethanol for further examination.

A deliberately limited number of morphological characters were examined in this study, with the aim of proposing a quick method to distinguish the larvae of *C. zealandica* and *C. brunneum*, rather than providing an extended morphological description of the species. Characters frequently used in scarab larval taxonomy were targeted (e.g., Hoy & Given 1952; Mico et al. 2001; Dittrich-
Schröder et al. 2009, Garcia et al. 2009, Sipek & Ahrens 2011). The labrum, epipharynx, mandibles and the structures of the raster (Figure 4.1) of the species were examined and compared using a stereomicroscope.

Figure 4.1. Lateral aspect of generalised *Costelytra* sp. 3rd instar larva. EP, epipharynx; L, labrum; M, mandible; R, raster. Scale 1mm.

Several characteristics of the septula of the raster were further analysed with the aim of selecting informative indicators for species diagnosis. Measurements considered were the minimum and maximum aperture size of the septula, the number of pali, the length and the width of the septula (Figure 4.2). This analysis was conducted using t-tests with R software (R Development Core Team 2009).
Figure 4.2. Generalised septula of the raster of *Costelytra* sp. indicating (a) measurements used for specimen identification and their photographs in (b) *C. zealandica* and (c) *C. brunneum*. MAX A, maximum aperture; MIN A, minimum aperture; RL, raster length; RW, raster width; P, pali. Scale 0.1mm.

4.3. Results

4.3.1. Molecular identification of the larvae

The cryptic appearance of the third instar larvae collected was similar for the 24 specimens and matched Hoy and Given’s (1952) description type of *C. zealandica* larva.

However, the molecular analyses by multiplex PCR allowed the identification of 12 larvae as *C. zealandica* and 12 as *C. brunneum* that, respectively, displayed COI fragment sizes of 546-bp and 304-bp.

4.3.2. Morphological characters

4.3.2.1. Labrum

The labrum was slightly asymmetrical in both species (Fig. 4.3.a and 4.3.b). The labrum of *C. brunneum* (Figure 4.3.a) has a medial projection with a pair of distinct lateral teeth and a pair of strongly projecting teeth on the antero-lateral margin. In contrast, *C. zealandica* (Figure 4.3.b),
has a medial projection with, at best, a pair of faint lateral teeth and a pair of faint teeth on the antero-lateral margin.

Figure 4.3. The labrum (a, b) and epipharynx (c, d) of *Costelytra brunneum* (top) and *C. zealandica* (bottom). With (i) angular anterolateral shape, (ii) zygum widely connected to epizygum, (iii) epizygum and heli. Scale 0.1mm.

4.3.2.2. Epipharynx

In *C. brunneum*, the epipharynx presented a more angular anterolateral shape than in *C. zealandica* (Figures 4.3.c and 4.3.d) and was composed of up to four projections (Figure 4.3.d). In addition, the epipharynx of *C. brunneum* consistently had no more than four long and dark coloured heli (Figure 4.3.d), whereas *C. zealandica* larvae always had five or more as described by Hoy and Given (1952). Hoy and Given (1952) described the zygum as incomplete in *C. zealandica*, in contrast, the right zygum was complete and widely connected with the epizygum in *C. brunneum* (Figure 4.3.d).
4.3.2.3. Mandibles

No differences in mandible morphology were detected; they were consistently strong, in shape, light brown basally and dark apically and both were consistent with the description of Hoy and Given (1952) for *C. zealandica*.

4.3.2.4. Raster

In both species, the septula of the raster comprised a ring of pali that was anteriorly closed but with an aperture at the anal end (Figure 4.2). In *C. zealandica* (Figure 4.2.b), the septula was subcircular, whereas in *C. bruneum* (Figure 4.2.c) it was curved laterally but formed an acute angle anteriorly. When analysed individually, none of the septula measurements alone was species-diagnostic. However, in pairwise comparisons, the combination of septula length and septula width displayed non-overlapping distributions for the two species (Figure 4.4), therefore is diagnostic.

![Diagram of raster septula measurements for larvae of *Costelytra zealandica* (closed circles) and *C. bruneum* (open circles). Each circle corresponds to one individual. Grey areas correspond to overlapping closed and open circles. All measurements are in mm apart for the number of pali (number of setae).](image)

**Figure 4.4.** Pairwise comparisons of raster septula measurements for larvae of *Costelytra zealandica* (closed circles) and *C. bruneum* (open circles). Each circle corresponds to one individual. Grey areas correspond to overlapping closed and open circles. All measurements are in mm apart for the number of pali (number of setae).
The ratio of these two measurements was significantly different between the two species (t-test, t = 8.98, df = 20.21, p < 0.001). It was consistently over 1.279 for *C. bruneum* and below 1.247 for *C. zealandica* and because there is no overlap in these values, this ratio may be used as a species diagnostic. In addition, this ratio was representative of the general appearance of the species septula, with higher values for the *C. zealandica* rounded shape and lower ones for *C. bruneum* oval shape. The other parameters measured all displayed overlapping values between the two species (Figure 4.4) and are therefore not diagnostic.

### 4.4. Discussion

This study showed that three morphological characters (i.e., raster, labrum and epipharynx) were sufficient to accurately distinguish between the larvae of the two closely related scarab species *C. zealandica* and *C. bruneum* (Table 4.1). This confirms that a few carefully chosen morphological features can allow easy distinction between two closely related species, as observed for many other scarab larvae (e.g., Mico et al. 1999; Dittrich-Schröder et al. 2009; Garcia et al. 2009). Even taken individually, each of the characters described in this study can assist with a preliminary species identification in the field providing a practical tool for non-specialists.

#### Table 4.1. A comparison of the three morphological characters selected for distinguishing between the larvae of *Costelytra zealandica* and *C. bruneum*.

<table>
<thead>
<tr>
<th>Morphological character</th>
<th><em>Costelytra zealandica</em></th>
<th><em>Costelytra bruneum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Labrum</td>
<td>Medial projection with, at best, a pair of faint lateral teeth; with a pair of faint teeth on the antero-lateral margin</td>
<td>Medial projection with a pair of distinct lateral teeth; with a pair of strongly projecting teeth on the antero-lateral margin</td>
</tr>
<tr>
<td>Epipharynx</td>
<td>Margin roughly convex and apex slightly crenated and distinctly projected; irregular antero-lateral shape; five or more heli; incomplete zygum connected to the epizygum</td>
<td>Margin and apex deeply crenated and indented; very angular antero-lateral shape with up to four projections; no more than four long heli; complete right zygum widely connected to the epizygum</td>
</tr>
<tr>
<td>Septula of the raster</td>
<td>Anteriorly closed with wider aperture at the anal end; short and large rounded shape</td>
<td>Anteriorly closed with narrower aperture at the anal end; long and narrow oval shape</td>
</tr>
</tbody>
</table>

Note: 1Based on Hoy & Given 1952

The septula characters that allow a clear distinction between the two species are a longer and narrower septula, in *C. bruneum* than in *C. zealandica* (Figures 4.2.b; 4.2.c; 4.4). Because these are external and easily accessible morphological characters, they can be assessed using a hand...
lens, and so provide a reliable tool for distinguishing these species without the need to dissect mouthparts or the requirement of access to fragile and expensive microscopy materials in the field. For difficult or ambiguous cases, a simple calculation of the ratio of the septula length to the septula width in the laboratory would then allow a definitive species identification. In this study, the threshold of this ratio was based on the examination of 24 individuals; further measurements would contribute to refine it if needed (e.g. examination of further populations across wider geographical ranges). Although it is unlikely that the general rounded vs oval shapes of the septula varies across a wider geographical range, it is possible that inter-population variations occur. Indeed, slightly bigger adults *C. zealandica* have been reported in the North Island (Travis Glare, personal communication, Bio-Protection Research Centre NZ), which suggests that their larval stages could also be bigger with slightly larger septula measurements. However, this would not affect the ratio septula length/ septula width used here. Additionally, the septula features described in this study can be non-invasively examined to distinguish between live larvae of the two species. Such non-invasive identification methods are compatible with ecological and behavioural studies.

Combining a molecular approach with traditional taxonomy was highly successful in identifying characters to differentiate the larvae of *C. zealandica* and *C. brunneum*. This technique can be readily applied to other difficult-to-separate scarab larvae but it is also applicable to other insects, as recently reported by Damm et al. (2010) in dragonflies. Furthermore, early detection, is often crucial in pest-management (Britton et al. 2010), and depends upon correct identification of specimens. Efficient pest management programmes often require ecological and behavioural assessments of the target species (Dick 2008; Jang et al. 2009), which are likely to involve sampling a fairly large number of specimens from the field and require a rapid means of assessing their identity. This study has succeeded in identifying simple morphological characters for live specimens that can be used in the field to distinguish between the two model species of this thesis.
Chapter 5

The implication of differential feeding preferences and fitness performance in *Costelytra zealandica* and *C. brunneum* on native and exotic hosts

*Results of this chapter submitted as*


*Abstract*

It is acknowledged that the widespread replacement of native ecosystems by productive land creates new and complex relationships among the flora and fauna that are affected by this anthropogenic-driven process. Although detrimental for numerous species, the creation of new ecological conditions by land modification sometimes results in the outbreak of a species, belonging to the native community that can then quickly reach the status of native pest or invader. In New Zealand, the introduction of exotic pastoral plants has resulted in the alteration of the diet of the native coleopteran *Costelytra zealandica* (White) such that this insect has achieved the rank of invasive pest. In contrast, *C. brunneum* (Broun), a closely related congeneric species, has not developed such a strong relationship with these new host plants. This study aimed to investigate the feeding preferences and fitness performance of these two coleopteran species, to increase fundamental knowledge about the mechanisms responsible for the development of invasive characteristics in native insects. To this end, (1) the survival and growth performance of two distinct populations of *C. zealandica* and one of *C. brunneum* were tested using a selection of four native and exotic host plants, (2) the effect of these various host plants, on larval fitness response under different temperature regimes was investigated, and, (3) the response of larvae of both *Costelytra* species to choice tests where they were given a choice between a native and an exotic host plant, was tested. The results of these experiments showed that the pest *C. zealandica*, when sampled from exotic pastures, does not seem able to fully utilise its ancestral native hosts and shows better fitness performance on exotic hosts at 15°C, particularly on clover. In contrast, the individuals sampled from native grasslands did not display better performance on one host or another but, interestingly, showed similar feeding preferences to their non-pest congenerics *C. brunneum*. This study corroborates the existence of a strong intra-specific variation in the diet of the pest species *C. zealandica*, reflecting an evolutionary process (i.e. host-shift or host range expansion) and putative abilities (i.e. pre-existing ability to use ‘new’
hosts), which offers new insights for investigation of some mechanisms underpinning the establishment of invasive characteristics in this species.

5.1. Introduction

By widely replacing native ecosystems by lucrative productive land, modern intensive agriculture has often been regarded by ecologists as a driver for substantial biodiversity loss (Robinson & Sutherland 2002, Tilman et al. 2002, Foley et al. 2005). Although detrimental for numerous species, by creating ‘new’ ecological conditions, these anthropogenic modifications appear to be beneficial under certain circumstances for some species of the native pool. For instance, it is acknowledged that the high diversity of phytophagous insects partially depends upon evolutionary processes that occur through the action of factors affecting their diet breadth (Gaete-Eastman et al. 2004), like the introduction of a new host plant. Hence, the repercussions of anthropogenic-driven modification(s) on native ecosystems are worth investigating as they can provide valuable clues in the understanding of the insect invasion process. In addition, the comparison of native and invasive congeners is recognised as a useful approach for identifying characteristics that promote invasiveness (Munoz & Ackerman 2011), even more so, as in this study, when the ‘invasive congener’ is native itself.

In New Zealand, the introduction of exotic pastoral plants has resulted in the alteration of the diet breadth of the native coleopteran *C. zealandica*, resulting in the larvae of this endemic insect to feed intensively on ryegrass and white clover and being ranked as a major economic pest (Pottinger 1975, Richards et al. 1997). Interestingly and in contrast, *C. brunneum* has not developed this habit and remains mostly distributed in native habitats (Given 1966, Lefort et al. 2012, 2013). Both species share similar native hosts, mainly comprising tussock species belonging to the Poaceae family commonly found in New Zealand native grasslands, and also occur sympatrically geographically (Given 1966, Lefort et al. 2012, 2013).

The present chapter aimed to investigate the feeding preferences and fitness response of these two coleopteran species, to provide new insights to investigate the mechanisms underpinning the invasion process in *C. zealandica* and ultimately in phytophagous insects in general. The specific objectives were to, (1) test the feeding, survival and growth performance of two distinct populations of *C. zealandica* when exposed to a selection of four native and exotic host plants, (2) investigate the effect of these various host plants on larval fitness response under different temperature regimes, and (3) perform choice tests where the larvae of both *Costelytra* species were given a choice between a native and an exotic host plant.
5.2. Material and methods

5.2.1. Insect sampling and plant culture

Four of the selected larvae sample sites (Section 2.2.1) were used in this study, Lincoln and Hororata, respectively labelled as sites A and B and Cass and Castle Hill as sites C and D. Extensive taxonomic assessments of the plants present on each site were not performed. However, the two highly dominant groups of plants present (i.e. visual estimation was over 80%) on sites A and B appeared to be exotic ryegrass and clover plants, while sites C and D were dominated by native tussock and fescue plants. Larval collection was carried out as described in Chapter 2 and all the collected larvae were tested for amber disease (Section 2.2.3.1). Healthy larvae were identified morphologically to the species level based on the morphology of the raster as described in Chapter 4 and in Lefort et al. (2013).

The exotic host plants Trifolium repens (white clover) and Lolium perenne (ryegrass), and the native plants Poa cita (silver tussock) and Festuca novae-zealandiae (fescue tussock) were grown following the methodology described in Chapter 2 (Section 2.2.3).

5.2.2. Costelytra zealandica fitness performance on various host plants and under different temperature regimes

Third instar larvae of C. zealandica collected from sites A (population A, n=430) and C (population C, n=90) were randomly allocated to the four different feeding treatments (white clover, ryegrass, silver tussock or fescue tussock). Each feeding treatment was conducted at three different temperatures (10, 15 and 20°C) for population A and only at 15°C for population C due to smaller total sample size. Experimental temperatures were selected in accordance with the soil temperature range under which third instar larval of both species occur in New Zealand (Figure 5.1), either within or at its upper limit. Indeed, vertical movements in Costelytra larvae are known to be limited to the 10 first centimeters of the soil column, as a consequence and based on the assumption that both Costelytra species follow a univoltine life cycle (Figure 2.2), actively feeding third instar larvae are therefore only rarely exposed to soil temperatures as low as 10°C or as high as 20°C. Therefore, 10 and 20°C were chosen to represent realistically challenging temperatures for C. zealandica and C. brunneum, and are qualified as such throughout this thesis. Each larva was kept individually in a 35 ml plastic container containing 50 g of gamma-irradiated soil (Schering-Plough Animal Health, Wellington, NZ) and was fed ad libitum with chopped roots of the selected treatment plant (i.e. white clover, ryegrass, silver tussock or fescue tussock). Containers were randomly arranged on plastic trays and kept in an incubator under the different temperature regimes.
The experiment was conducted over 17 weeks, after which all larvae were assessed for survival. During the first 6 weeks of the experiment, larvae were weighed weekly. Statistical analyses on the effect of plant species diet and temperature on larval survival were carried out using a Chi-squared test. Growth data (i.e. weekly weight gain of the larvae) were analyzed by one-way analysis of variance (ANOVA), after exclusion of larvae that died before the end of the 6 weeks of data collection, with either host plant or temperature as factor. Statistical tests were conducted with R software (R Development Core Team 2009) and GenStat® (GenStat 14, VSN International Ltd, UK). Following the results of this experiment, one native and one exotic host plant were selected as “best host” (i.e. the native and the exotic host on which *C. zealandica* larvae displayed the best fitness performance) to perform the rest of the experiments presented in this thesis, in order to minimise number of experimental treatments and sampling effort associated.

### 5.2.3. *Costelytra* spp. feeding preferences – native vs exotic host choice test

The feeding preferences between native or exotic host plants of *C. zealandica* and *C. brunneum* larvae were tested using a three choice olfactometer. The olfactometer comprised three extended arms filled with gamma-irradiated soil (Schering-Plough Animal Health, Wellington, NZ) and a central exposure chamber to insert the larvae. At the end of each arm, a pot containing either no...
plant (control pot), white clover or silver tussock was connected. Third instar larvae of *C. zealandica* collected from sites B (population B, n=35) and C (population C, n=35) and *C. brunneum* (population D, n=35) from sample site D were used for this experiment (Section 2.2.1). For each population, the bioassay was replicated seven times, with five fresh larvae inserted together in the central exposure chamber. After 24 hours, pots were disconnected from the olfactometer device, emptied of their content and larvae were counted. Between each trial, all components of the olfactometer were thoroughly washed with warm water and left to soak in clean water overnight, finally being left to air-dry on a clean counter and reassembled. Statistical analyses were performed using paired t-tests. The significance of the choice or not of a plant was first tested (i.e. no plant (control + no choice) versus plants (white clover + tussock)). In the event that a plant was chosen, the significance of the plant choice itself was also tested (i.e. white clover versus tussock). Statistics were conducted with R software (R Development Core Team 2009).

5.3. Results

5.3.1. *Costelytra zealandica* performance - larval survival

5.3.1.1. Native host vs exotic host

Larval survival of *C. zealandica* was first assessed irrespective of temperature by combining the data across temperatures for each host plant treatment over the 17 weeks. In contrast with the larvae from native grasslands (population C), the larvae collected from exotic pastures (population A) displayed significantly better survival rates when fed with exotic host plants (24.0% survival) compared with larvae fed with native hosts (18.6% survival) ($\chi^2=9.3442$, d.f.=1, $p<0.01$) (Figure 5.2). The larvae from exotic pastures (population A), displayed significantly higher survival rates when fed with exotic host plants (i.e. clover and ryegrass) (>26%) compared to the other host plants (i.e. silver tussock and fescue tussock) (<22%) ($\chi^2=9.6176$, d.f.=3, $p<0.05$).
Figure 5.2. Percentage of larval survival of *Costelytra zealandica* population from sites A and C after 17 weeks of feeding treatment on native (dark grey bars) and exotic (grey bars) host plants. Sample sizes were respectively n=226 on natives and n=204 on exotics for population A (all temperatures combined), and n=46 on natives and n=44 on exotics for population C.

### 5.3.1.2. Temperature effect

After 17 weeks of treatment and for the population collected from exotic pastures (population A), when data for each temperature were considered separately (10, 15 and 20°C), survival differences were highly significant both on native and exotic plants ($\chi^2=87.9216$, d.f.=3, p<0.01 and $\chi^2=57.7089$, d.f.=3, p<0.01, respectively) and were significantly better at 10°C (Figure 5.3).

Figure 5.3. Percentage larval mortality of *Costelytra zealandica* population A after 17 weeks of feeding treatments on native (a) and exotic (b) host plants and overall survival at different temperatures (10, 15 and 20°C). Sample sizes were respectively n=76 at 10°C, n=72 at 15°C and n=78 at 20°C on native hosts and n=65 at 10°C, n=62 at 15°C and n=77 at 20°C on exotic hosts.
5.3.2. *Costelytra zealandica* performance - Larval growth

5.3.2.1. Native host vs exotic host

As for larval survival, larval growth of the larvae collected from exotic pastures (population A) was first compared by combining the data across temperatures for each host plant treatment. No treatment effect was detected for the population from native grasslands (population C), while the larvae from exotic pastures (population A) gained significantly more weight when fed on clover after 6 weeks compared to when they were fed with other host plants (ANOVA, p<0.001) (Figure 5.4).

![Figure 5.4](cumulative_weight_gains.png)

**Figure 5.4.** Cumulative weight gains of *Costelytra zealandica* population A after 6 weeks of feeding treatment on different host plants. Sample sizes were respectively n=88 on fescue, n=86 on tussock, n=91 on ryegrass and n=78 on clover (all temperatures combined). Vertical bars represent 5%LSD.

5.3.2.2. Temperature effect

The analysis of data at different temperatures revealed that the larvae from exotic pastures (population A) only displayed significantly higher growth on exotic host plants at 15°C at the end of the 6th week of treatment (ANOVA, P=0.009) (Figure 5.5).
Figure 5.5. Cumulative weight gain of *Costelytra zealandica* population A after 6 weeks of feeding treatment on native and exotic host plants at 10°C (a), 15°C (b) and 20°C (c). Sample sizes were respectively n=68 on native and n=59 on exotic hosts at 10°C, n=63 on native and n=55 on exotic hosts at 15°C and n=43 on native and n=55 on exotic hosts at 20°C. Vertical bars represent 5%LSD.

5.3.3. *Costelytra* spp. feeding preferences – native vs exotic host choice test

In the choice test only *C. zealandica* collected from exotic pastures (population B) displayed a preference for the selected exotic host plant (white clover) ($t$-test, $t = -7.8127$, df = 6, $p <0.01$) (Figure 6). In contrast, *C. zealandica* collected from native grassland (population C) and *C. brunneum*, did not show a preference for any plant (Figure 5.6).

Figure 5.6. Choice test and feeding preferences of three populations of *Costelytra* larvae using one exotic (white clover) and one native (silver tussock) host. With in (a), *C. zealandica* population B preferences, in (b) *C. zealandica* population C preferences and in (c) *C. brunneum* population D preferences.
5.4. Discussion

This chapter firstly explored the fundamental variation in *C. zealandica* feeding preferences and fitness response to various hosts. The results presented corroborate the existence of a strong intra-specific variation of the diet breadth of this pest species. This study also demonstrated similarities between the feeding preferences of a population of *C. zealandica* collected from an isolated native habitat with those of the congeneric non-pest species *C. brunneum*, giving rise to further questions about the mechanism(s) underpinning the process of invasion in *C. zealandica* into improved pastures throughout New Zealand. Finally, this study demonstrated the potential importance of soil temperature on the exploitation of exotic pastoral host plants by the invasive species *C. zealandica*.

The overall fitness performance, as measured by survival and growth, of *C. zealandica* collected from exotic pastures was better on exotic host plants, particularly on clover. Inheritance on host choice (Mousseau & Dingle 1991, Mousseau & Fox, 1998), where offspring display high fitness performance (Fox 2006) and similar host preferences as their mother (Craig et al. 2001), is a possible explanation. Similarly, a maternal effect, such as the ‘mother knows best principle’, which suggests that females tend to oviposit on host plant(s) that can potentially increase their offspring survival (Scheirs et al. 2000, Mayhew 2001), is also a possible explanation. These hypotheses are supported by the results of the choice test, where this particular population, which is likely to have been feeding on exotic pasture plants for several generations, clearly chose clover as the preferred host plant. In contrast, the population of *C. zealandica* collected from their native range did not display better fitness performance on any host, nor displayed preferences during the choice tests. This observation refutes the hypothesis of inheritance and maternal effect on host choice mentioned earlier, since based on this principle, this population would have been expected to prefer its native host (i.e. tussock) and have better fitness performance on this plant compared with any other host (e.g. white clover). White clover was the only legume used in this study, which may partially explain the differences in larval weight gain observed in the *C. zealandica* population collected from exotic pastures. Because of their bacterial symbiosis resulting in an ability to fix nitrogen (Awmack & Leather 2002), the nutritional value of this family of plants is likely to be higher than that of the grasses used as alternative hosts in this study. However, this alternative hypothesis does explain the response of the other *C. zealandica* population studied, which would have been expected to show higher weight gain on clover as well.

Based on the similar survival rates observed in the two populations of *C. zealandica* studied, and taking into consideration the fact that the population collected from native grassland was presumably isolated enough to have never encountered exotic hosts prior to the experiment, it appears that the use of exotic plants by this species is likely a pre-existing ability. Diegisser et al.
(2009) and Ding and Blossey (2009) both suggested the possible requirement of insect pre-adaptation for the exploitation of a novel host plant. The similarity of host choice, observed between *C. zealandica* collected from native grassland and *C. brunneum*, along with the current difference of exploitation of exotic pastoral plants by the two species, reinforces the possibility of some degree of pre-adaptation in *C. zealandica*. Even so, the nature of such a trait has yet to be determined for the species studied.

From another perspective, the defence mechanisms employed by the different host plants and their effect(s) on the fitness performance of the insect species studied may be questioned. In a recent review about phytophagous insects and plant defences, Ali and Agrawal (2012) reaffirmed that generalist insects do not master, and therefore do not totally overcome their host defences, but possess ‘general mechanisms’ to tolerate an array of those defences. It is possible to observe variations in this tolerance, particularly when the host-range utilised by the insect is highly diversified and, consequently, when the family of plants have differential evolutionary histories that may have resulted in slight variations in their defence mechanisms. Here, *C. zealandica* may have been, in terms of fitness performance, less affected by the defences of white clover compared to those of the other hosts or, conversely, may have somehow benefited from the defences of this particular host. The latter phenomenon has been observed several times in recent insect-host interaction studies, where the defences of the hosts were artificially triggered and the resulting fitness response of the insects studied were unexpectedly enhanced (e.g. Chapter 7, Pierre et al. 2012, Robert et al. 2012).

Apart from the influence of the host itself, it is widely recognised that abiotic factors play key roles in overall insect performance and regulate the ecology of many insect communities (Savopoulou-Soultani et al. 2012). Temperature directly affects insect development, survival and abundance (Bale et al. 2002). In this study *C. zealandica* larvae showed higher survival rates at lower temperatures even though their development, in terms of weight gain, was depressed. These results were irrespective of the host plant, and are consistent with the fact that developmental time in insects is slower at low temperatures because of the increased duration of cell division (Van Der Have & De Jong 1996, Folguera et al. 2010). The higher survival rates observed at lower temperature might have a proximal basis in the inhibiting effect that low temperatures could have on pathogens of *C. zealandica*. In this study, larvae potentially affected by amber disease, the most common disease encountered in *C. zealandica* (Jackson 1990), were discarded. However, other natural enemies, either bacterial, fungal of protozoan (Jackson 1990, Glare 1992), could have been present in some of the remaining larvae, explaining the higher mortalities observed at higher temperatures. Interestingly, a significant increase in fitness performance observed in terms of weight gain for larvae feeding on exotic host plants, occurred only at 15°C. As demonstrated by Ding & Blossey (2009), insect populations on different host plants can display distinct habitat preferences; it appears in this study the use of exotic plants as hosts was
facilitated by an average soil temperature of 15°C. Again, the presence and effect of C. zealandica natural enemies could have been partly responsible for these observations. In this study, contamination may have occurred by an accidental introduction of the pathogen with the native host plants given as a feeding treatment to the larvae, or through the survival of microbial pathogenic propagules on the cuticle of collected larvae. However, this explanation does not support the similar fitness performance observed for C. zealandica population A on exotic and native hosts at a higher temperature (i.e. 20°C) and, given the randomization of the treatment among the larvae, it is quite unlikely that only those kept at 15°C were contaminated.

Fifteen degrees was selected as representative of an average soil temperature encountered throughout most part of New Zealand during the intensive feeding stages of Costelytra larvae (i.e. second and early third instars). The highest soil temperature tested (i.e. 20°C) is rarely encountered in New Zealand soils at the depths that the larvae usually occur, except in the more northern locations of the North Island. The low survival at 20°C would explain why C. zealandica is not considered a major pasture pest in the area north of Waikato (East et al. 1981). The latter finding might be of particular importance in the current context of climate change, where variation in soil temperatures may influence and inflect the ability of C. zealandica to benefit from exotic pasture hosts more than from native plants found in its native range, and unseat this species from its status of major pasture pest. As mentioned earlier, if an ability in this insect species to benefit from the defences of a particular host exists, it is likely that temperature plays an important role in this process. As established by Bale et al. (2002), the successful completion of a life cycle in a phytophagous insect is representative of its ability to adapt to its host(s) and to the climatic environment in which they are found. The invasion success of the larval stage of C. zealandica certainly reflects the extent of this capability. Furthermore, the observation of significant intra-specific variation in the diet of this species, which might reflect a host-shift or a host range expansion, offers new insights to further investigate the establishment of potential mechanisms underpinning the invasive characteristics of this species. These latter points will be discussed more in detail in Chapter 6.
Chapter 6

Host-shift vs. host range expansion in *Costelytra zealandica*, a potential driver of invasion success

**Results of this chapter submitted as**

Lefort M.-C., Boyer S., De Romans S., Glare T.R., Armstrong K and Worner S.P. (currently under revision) Invasion success of a scarab beetle within its native range: host range expansion vs. host-shift. *PeerJ*

**Abstract**

Only recently has it been formally acknowledged that native species can occasionally reach the status of 'pest' or 'invasive species' within their native range. The study of such species has potential to help unravel fundamental aspects of biological invasions. A good model for such a study is the New Zealand native scarab beetle, *Costelytra zealandica* (White) which, even in the presence of its natural enemies, has become invasive in exotic pastures throughout the country. Because *C. zealandica* still occurs widely within its native habitat, this species might has only undergone a host range expansion onto exotic hosts rather than a host shift. Moreover, this host range expansion, rather than enemy release, could be one of the main drivers of its invasion success. This chapter investigates the fitness response, as measured by survivorship and weight gain, of populations of *C. zealandica* from native and exotic ecosystems, to several feeding treatments comprising its main exotic host plant as well as one of its ancestral hosts. These results suggest that the initial established hypothesis was incorrect and that *C. zealandica* populations occurring in exotic pastures have experienced a host-shift (loss of fitness on the ancestral host) rather than simply a host-range expansion (ability to equally use both ancestral and a new host). This finding suggests that an exotic plant introduction can facilitate the evolution of a distinct native host-race, a phenomenon often used as evidence for speciation in phytophagous insects and which here may have been instrumental to the invasion success of *C. zealandica*.

**6.1. Introduction**

Plant introductions to novel habitats have occurred worldwide over hundreds of years to sustain human migrations and subsequent needs (Burnett et al. 2012). Even today, the number of such
introductions continues to increase, although attention has changed over recent decades from species that mainly sustain food production (Godfray et al. 2010) to species that are introduced accidentally (McNeill et al. 2011) or planted for amenity purposes (Brasier 2008). As a result, a large variety of more or less complex relationships with the members of native communities have flourished (for a review see Cox 2004). Although these interactions often result in population declines among the native community (Ding & Blossey 2009), sometimes the introduction of exotic plants provides an opportunity for a species to expand and flourish outside of its native habitat. This can occur by the process of host range expansion (Mack et al. 2000) and ultimately of host-shift, sometimes referred in the literature as host-switching (Agosta 2006) or host-transference (Holder 1990). Agosta (2006) defines a host-shift as the continuation of a host range expansion whereby a population of a phytophagous species forms an association with a novel host plant. In addition, Diegisser et al. (2009) specified that, in this process, the population shifting might not be able to use its new and its ancestral host simultaneously, which can be detected by a host-plant associated fitness trade-off on the ancestral host (Via 1990, Diegisser et al. 2009). In contrast, host-range expansions do not result in such fitness compromises, allowing the population to use both its new and ancestral hosts (Diegisser et al. 2009) without generating detrimental fitness responses. It might be expected to occasionally observe these types of response in native insects that sometimes reach the status of ‘pest’ or ‘invasive species’ on introduced plants.

In the last few years, Valéry et al. (2008a, 2008b, 2009, 2013) debated the terminology relative to ‘biological invasion’ and demonstrated that it should not be solely confined to allochthonous species. For insects alone, and with more than 60 native species that have become notable for the economic damage that they cause (Scott 1984), New Zealand is a perfect illustration of this assertion. In this country, the larval form of the native scarab *C. zealandica* is certainly one of the most well known local pests that attack numerous exotic plants (Given 1966, East & Pottinger 1984, Scott 1984, Grimont et al. 1988, Richards et al. 1997), among which are several European-style pastoral plants such as clover and ryegrass. Despite this apparent success on exotic hosts, this species still occurs widely within its native habitat, which is mainly composed of local fescue and tussock species. The present chapter aims to investigate whether the rise of *C. zealandica* as a native biological invader was driven by just a host range expansion rather than by a complete host shift. The fitness response of two populations of *C. zealandica* was investigated based on the survivorship and weight increase of third instar larvae under several feeding treatments comprising an exotic host plant as well as one of its ancestral hosts.
6.2. Material and methods

6.2.1. Insect sampling and plant culture

To undertake this study only two sample sites from the South Island of New Zealand were used, a typical exotic pastoral site in Hororata (site B, see Section 2.2.1) and a native grassland in Cass (site C, Section 2.2.1). Larvae from these sites were labeled population A and B, respectively in the present study. Standard collection and identification methods, as well as the amber-disease free test, as described in Chapter 2, 3 and 4 of this thesis were used for all the collected larvae. All larvae were then randomly assigned to the various experimental treatments.

Based on the result of the fitness performance experiment performed in chapter 5, the exotic host Trifolium repens (white clover) the native host Poa cita (silver tussock) were selected for this study and grown following the methodology described in Chapter 2 (Section 2.2.3).

6.2.2. Native vs exotic hosts and artificial host-shift experiment

Following identification, C. zealandica larvae (n=180) were weighed and placed in individual 35 ml plastic containers containing 50 g of gamma-irradiated soil (Schering-Plough Animal Health, Wellington, NZ). Containers were randomly allocated to three trays to create 10 blocks, where the larvae were ordered from the lowest to the highest weight on the trays to allow the detection of potential effect of confounding factors. Each container was randomly assigned to a feeding treatment. Feeding trials were performed at 15°C over a period of 12 weeks corresponding to the most intense feeding period of the third instar larval stage in C. zealandica. Larvae were fed ad libitum with freshly chopped roots of the selected host plant. They were either fed with clover or tussock for 12 weeks respectively for treatments 1 (T1) and 2 (T2), or with tussock for 7 weeks followed by a shift of 5 weeks on clover for treatment 3 (T3).

The fitness response of the larvae was evaluated by measuring survivorship and percentage increase in weight on a weekly basis. All statistical tests were conducted with R software (R Development Core Team 2009) and GenStat® (GenStat 14, VSN International Ltd, UK).

Statistical analyses on the effect of each host plant (T1 and T2) and of the artificial host shift (T3) on larval survival were carried out using a Chi-squared test. The treatment effect (T1, T2 and T3) on larval growth was analysed by analysis of covariance (ANCOVA), with the initial weight of the larvae used as a covariate. The latter analysis was performed after exclusion of larvae that died before the end of the 14 weeks of data collection.
6.3. Results

6.3.1. Larval survival

The larvae collected from exotic pastures (population A) displayed significantly better survival rates when fed with the exotic host plant (T2, 86% survival) as opposed to their native host (T1, 20% survival) ($\chi^2=86.6364$, d.f.=1, $p<0.001$) (Figure 6.1). Similarly, these larvae survived significantly better when fed with a combination of native followed by exotic host plants (T3, 56% survival) than when fed with their native host only (T1) ($\chi^2=26.9118$, d.f.=1, $p<0.001$).

In contrast, no significant survival differences were detected for the larvae collected from native grasslands (population B) across all treatments (Figure 6.1) (Chi-squared tests respectively T1/T3 $\chi^2=3.1765$, d.f.=1, $p=0.07471$, and T2/T3 $\chi^2=0.8985$, d.f.=1, $p=0.3432$).

![Figure 6.1](image)

**Figure 6.1.** Percentage of larval survival of two populations of *Costelytra zealandica* (n=120) following 12 weeks of feeding treatment with tussock (T1), clover (T2) or with a combination of the two later plants (T3). Population A (black bars) were collected from exotic pastures and population B (grey bars) were collected from New Zealand native grasslands. Chi-squared tests indicated that a/a and b/c survival rate differences were highly significant (respectively $p<0.001$ and $p<0.01$) and that b/d and c/d differences were not significant ($p>0.05$).

6.3.2. Larval growth

When the larvae were exposed to the artificial host-shift feeding treatment (T3), and fed with native tussock during the first phase of the experiment, no differences in terms of weight gain were detectable between the two populations studied (Figure 6.2). However, this trend changed considerably after the host-shift that occurred in week 7. Larvae belonging to the population
collected from exotic pastures (population A) quickly increased weight by over 40% during the second phase of treatment that lasted for 5 weeks, which was significantly more than population B larvae that only increased their weight by about 16.5% (Figure 6.2).

![Figure 6.2](image)

**Figure 6.2.** Cumulative weight gain of two populations of *Costelytra zealandica* larvae following 12 weeks of artificial host-shift feeding treatment (T3), where larvae were fed for 7 weeks on tussock and 5 weeks on clover. Population A (dark grey line) (n=17) collected from exotic pastures and population B (light grey line) (n=24) collected from New Zealand native grasslands. Vertical bars represent 5%LSDs at the end of each week of treatment.

It appeared that population A responded much better to the exotic host feeding as shown by the rapid increase in weight just after the host-shift in T3, and also by an overall weight gain close to 60% for the larvae submitted to T1 (Figure 6.3). In contrast, when population A was kept feeding on native tussock for 12 weeks (T2), larvae lost a significant amount of weight (Figure 6.3). From week 8 onward, the differences between this treatment (T2) and the exotic based treatments (T1 and T3) were highly significant (all weeks, ANCOVA, p values < 0.001) (Figure 6.3).
Figure 6.3. Cumulative weight gain of *Costelytra zealandica* larvae collected from exotic pasture (population A) following 12 weeks of feeding treatment on various host plants. Native tussock feeding treatment (T1) in dark grey (n=6), clover feeding treatment (T2) in light grey (n=26) and artificial host-shift feeding treatment (T3) in medium grey (n=17). Vertical bars represent 5%LSDs at the end of each week of treatment.

6.4. Discussion

An important challenge for ecologists and evolutionary biologists is to investigate the various contributing factors to biological invasions. Among these are the processes by which some species reach the status of invaders in their home range. The present study aimed to address the identification and investigation of such drivers in *C. zealandica*. The results of this study recorded the existence of strong intra-specific variation in fitness of this species. The variation was expressed as important differences in survivorship and weight increase when different larval populations, recovered from different host plants and regions, were exposed to their ancestral native or exotic host plants.

An overall high fitness performance was observed on clover, expressed as high survivorship and high larval weight increase, by *C. zealandica* collected from exotic pastures. As discussed in Chapter 5, such results may reflect some sort of inheritance and maternal effect (Mousseau & Dingle 1991, Mousseau & Fox, 1998), where the offspring of a given population is expected to display high fitness performance (Fox 2006) and similar host preferences as their parents (Craig et al. 2001). However, for this particular species, neither a genetic nor maternal effect, or an alternative explanation such as the high nutritional value of clover (Awmack & Leather 2002), can explain the observed increased performance of the larvae (Chapter 5). Nevertheless, it is quite likely that intrinsic mechanisms relying on a high degree of phenotypic plasticity, such as variation
in host tolerances (Agrawal 2000, Kant et al. 2008) rapid adaptation (i.e. evolutionary host-shift) (Holder 1990, Menken & Roessingh 1998, Agosta 2006) or ecological fitting sensu Agosta (2006) (i.e. ecological host-shift), might be partially or totally responsible for the high fitness performance observed in *C. zealandica* collected from exotic pastures and fed on clover. Agosta (2006) defined the term ecological host-shift as a process that occurs through that of a host range expansion, whereby an organism is able to use new resources at the moment of contact because of a latent ability that results in a novel association of species, and where consequently evolution by either member of the association shall not be a prerequisite. Because all the larvae of *C. zealandica*, regardless of their origin, displayed high survival rates when fed with clover as a ‘new’ host, this latter explanation appears appropriate. Furthermore, Holder (1990) suggested that this type of association often arises because of the physical proximity of the ancestral and the new host-plant species, a scenario that followed the European settlement in New Zealand, when numerous native forests and grasslands were replaced by exotic pastures and crops (McDowall 1994, Lee et al. 2006). Effectively, this pattern of early settlement modification of the New Zealand landscape resulted in new ecological configurations where native grasslands ended up neighboring exotic cultures and grass pastures. It is believed that this physical proximity has resulted in the contraction of native plant distribution ranges and in the exploitation of these new modified habitats by native species (Yeates 1991), as possibly observed in *C. zealandica* as an ecological host-shift.

Another tangible explanation for the exploitation of both native and newly exotic host plants by *C. zealandica* could be that this species has not -yet- undergone a host shift and but only a host-range expansion onto exotic pastoral plants. Such explanation is seemly because of the close relationship that exists between this process and that of an ecological host-shift, and where, in both cases, no significant adaptation to the newly encountered exotic host is required (Diegisser et al. 2009). However, the differences in fitness performance between the two populations of *C. zealandica*, which were observed following the ancestral host feeding treatment, negate this possibility and suggest another explanation. The larvae originating from exotic pastures seem no longer able to properly benefit from their ancestral host, as shown by very high mortality rates and low weight increase of the surviving larvae of this population. This fitness compromise, which is expressed as a host-plant associated fitness trade-off (Via 1990, Diegisser et al. 2009) resulting in some degree of maladaptation to the ancestral host plant of this species, is not compatible with the solely host range expansion theory and reinforces that of a host-shift occurrence (Diegisser et al. 2009) for the population originating from exotic pastures.

Even though the ecological host-shift theory appears to be supported by this case study, the slight variation in terms of weight gain between the two populations, following the artificial host-shift on clover suggests that some level of evolutionary change has occurred for the population collected from exotic pastures. Heard and Kitts (2012) suggested that a host-shift can be followed
by host-associated differentiation that can result in the evolution of new biotypes of specialist races, or so-called host-races (Diehl & Bush 1984, Drès & Mallet 2002). Over recent decades, numerous examples of host-race formation in insects have been described. Amongst the most recent examples, Downey and Nice (2011) reported the possibility of ongoing host-race formation in the juniper hairstreak butterfly (*Callophrys gryneus*), following the observation of differential larval fitness performance when reared on natal versus alternate hosts. The results of the present study strongly suggest a similar scenario, where an ecological host-shift in at least one population of *C. zealandica* would have led to the emergence of distinct host-races in this species. Hence, it is likely that the invasive *C. zealandica* might represent a particular biotype. Any phenotypic plasticity that initially facilitated the assumed host-shift and host-race formation, could, in the long term, lead to speciation (e.g. West-Eberhard 1989, Agrawal 2000, Agosta 2006, Heard & Kitts 2012) in this insect. Furthermore, these findings point to a very interesting case of sympatric host race formation facilitated by exotic plant introduction, and resulting in the emergence of a phytophagous insect to the rank of invasive species in its own native range.
Chapter 7

Responding positively to plant defences, a candidate key trait for invasion success in phytophagous insects

Results of this chapter submitted as


Abstract

Exotic plant introductions commonly result in modifications of ecological relationships in native communities. Occasionally, such introductions lead to the emergence of an invasive phytophagous insect from the native pool that can eventually become a serious pest. One hypothesis that can help explain the emergence of a pest in its native range is a pre-adaptation allowing the insect to break through the defences of a new host. In this chapter, this hypothesis was investigated by comparing the fitness responses of two New Zealand endemic scarabs when given a diet of an exotic pasture species, Trifolium repens, whose defences were artificially triggered by the phytohormone jasmonic acid. The scarab, Costelytra zealandica (White) has become a serious pest whereas its congener, C. brunneum (Broun), has not. Differential fitness responses were found between the larvae of the two species when exposed to a defence-induced diet. A significant weight increase was observed in the invasive species C. zealandica when fed with treated roots compared with untreated controls whereas no significant weight increase was observed in C. brunneum compared to the control treatments. This study suggests that the invasive species C. zealandica has a pre-existing ability to tolerate the defence chemicals of its exotic host and more interestingly, to benefit from them. This ability is possibly one reason why this species has become a serious pest of pasture throughout its native geographical range.

7.1. Introduction

An exotic plant introduction into a new environment can result in the establishment of novel relationships between the particular plant species or variety and the members of the native community (for a review see Cox 2004). The response of local species of phytophagous insects,
when faced with a new variety of introduced plant, sometimes counts among the most adverse of them. For instance pasture plants, which for most have meant to be cultivated over large areas, often border, overlap and may even replace the native habitats of indigenous species, providing ideal conditions such that a native insect reaches the status of biological invader. Yet, this result is not always the case. Insect responses to new plant introductions can greatly vary from one species to another, even when the insect species are closely related, for example belonging to the same genus, and share similar native habitats. Such an example is *C. zealandica* and *C. brunneum*, two endemic New Zealand scarabs, that have developed distinctive relationships with *Trifolium repens* (white clover), a temperate pasture species initially introduced into New Zealand because of its high economic value as livestock forage and green manure crop (Gillett & Taylor 2001, Badr et al. 2012). Indeed, the larval stage of *C. zealandica* quickly became a serious pest of white clover (Radcliffe 1971), while *C. brunneum*, although able to feed on the roots of this plant (pers. obs.), did not. The reason(s) for the differential response of the two species remain unclear, and potentially numerous ecological factors could explain them.

When facing attack from insects, many plants initiate the synthesis of inducible defence-related proteins upon their attackers (van Loon et al. 2006), a phenomenon sometimes referred as an immune response (Van der Ent et al. 2009) or plant immune-system (Erb et al. 2012). Several of these compounds are induced through the accumulation and the action of signaling molecules such as salicylic acid (SA), jasmonic acid (JA) or ethylene (ET) (van Loon et al. 2006, Lee 2009). For instance, trypsin-inhibitor is a serine proteinase inhibitor which is found widely throughout the plant kingdom (Koiwa et al. 1997), and which can be activated through the JA pathway (Cipollini et al. 2000). This protein belongs to a class of defence-related proteins that reduces the fitness of many phytophagous insects (Cipollini & Bergelson 2000), and consequently has the potential to greatly affect root feeders such as scarab larvae. Interestingly, a number of these inducible defence-related proteins have been detected in a congeneric species of white clover (for a review see Kigathi et al. 2009), the host plant selected to undertake this study.

The present chapter aims to determine if *C. zealandica*, in contrast to *C. brunneum* that has not proliferated like its congener, is pre-disposed to overcome the defences of white clover, hence increasing its fitness thereby contributing to its invasion success. To this end, larval populations of these two beetles species were submitted to a feeding experiment, involving an artificial induction of belowground defence compounds in their host. But, because the dynamic of inducible defence response in the belowground parts of plants is still poorly defined (Erb et al. 2012), this study also tested for the existence of an induced immune-response of white clover roots after a direct soil injection of the phytohormone JA.
7.2. Material and methods

7.2.1. Insect sampling and plant culture

Third instar larvae of *C. zealandica* from exotic ryegrass and clover pastures (Population 1, n=60) and from native tussock grasslands (Population 2, n=60) and *C. brunneum* from native tussock grasslands (Population 3, n=20), respectively from the sample sites B, C and D (see Section 2.2.1), were used for this experiment. Standard collection and identification methods, as well as the amber-disease free test, as described in Chapter 2 of this thesis were used for all the collected larvae.

Based on the result of the fitness performance experiment performed in Chapter 5, white clover was selected as exotic host and plants were grown from seeds as described in Chapter 2 (Section 2.2.3). Three weeks after sowing, pots were treated by direct soil injection of 5 ml of 10 µM JA (Sigma-Aldrich Chemical Co., St. Louis, Missouri), while controls were treated with solvent (0.6 ml of EtOH in 4.4 ml of distilled water). Plants were used for feeding 48 hours after treatment.

7.2.2. Evaluation of defence-induction in white clover roots

Semi-quantitative assessment of trypsin proteinase-inhibitor (TPI) induction, used as an indicator of the JA pathway activity, was carried out by radial diffusion assay (Jongsma et al. 1993). Briefly, roots of JA-treated clover and controls (n=24) were rinsed twice with distilled water. Sections (200 mg) of root material from individual pots were homogenized in liquid nitrogen and extracted with 175 µl buffer (100 mM Tris HCl and 10 mM CaCl₂). The extract was centrifuged for 2 min at 13,000g and 4°C. The supernatant (25 µl) was transferred to wells made into a gel containing bovine trypsin as substrate. These plates were prepared beforehand, as described in Jongsma et al. (1993) and Cipollini and Bergelson (2000). For each of them, 1.8 g of bacto-agar was added to 75 ml of TrisHCl, adjusted to pH 7.6 and autoclaved for 15 min. After cooling down to 40°C, 75 µl of bovine trypsin (1mg/ml in Tris-HCl at pH 7.6) was added. The preparation was poured into a Petri dish and the gel allowed to solidify at 4°C for 4 hours. Eleven wells, 4 mm in diameter were punched out of each agar plate to accommodate root extracts and positive controls comprising commercial soybean TPI in three concentrations (70 pM, 35 pM and 14 pM). The extracts were allocated randomly to the wells throughout the agar gel and were allowed to diffuse at 4°C for 18 hr.

After overnight incubation, staining was performed. Each gel was submerged for 2 min in a solution of 100 mM Tris-HCl, pH 7.6, containing 10 mM CaCl₂. Following what, a staining solution comprising 48 mg Fast Blue B Salt in 90 ml of 100 mM Tris Cl, pH 7.6, was mixed with 24 mg of *N*-acetyl-DL-phenylalanine-naphthyl ester in 10 ml of *N*, *N*-dimethylformamide, and immediately
poured onto the gel. The plates were incubated with the staining solution at 37°C for 30 min and rinsed under running water before examination.

TPI activity was visible as unstained circular inhibition zones around wells. Zone sizes were compared with those obtained from the three controls. All reagents were obtained from Sigma-Aldrich Chemical Co., St. Louis, Missouri. Statistical significance of the presence/absence of TPI activity was determined by exact binomial tests using the statistical software R (R Development Core Team 2011).

7.2.3. Costelytra spp. response to increased host defences

Larvae of each population were randomly allocated to two different feeding treatments. Each larva was kept individually in a 35 ml plastic container containing 50 g of gamma-irradiated soil (Schering-Plough Animal Health, Wellington, NZ) as per standard culturing method (Section 2.2.3.2). Containers were randomly arranged on plastic trays and kept in an incubator at 15°C. Over a period of six weeks, 70 larvae (n=30 for populations 1 and 2, and n=10 for population 3) were fed with JA treated clover roots, freshly chopped to avoid defence induction by the feeding larvae themselves, while another 70 (n=30 for populations 1 and 2, and n=10 for population 3) were fed with untreated clover roots. Larvae were fed ad libitum, and their weight and survival rates recorded weekly. The cumulative treatment effects on each population were analysed weekly by analyses of variance (one way ANOVA), while statistical analyses on the effect of induced host plant defences on larval survival were carried out using Fisher's exact tests. Statistical tests were conducted with GenStat® version 14.1 (VSN International Ltd., Hemel Hempstead) and R (R Development Core Team 2011).

7.3. Results

7.3.1. Evaluation of defence-induction in white clover roots

Trypsin inhibitor activity was detected on the agar plate by an evident variation in colouration (Jongsma et al. 1993, Cipollini & Bergelson 2000). The surrounding area of the wells, to which the root extracts of JA treated white clover were applied, remained clear while the rest of the gel stained a bright pink–purple color. Trypsin inhibitor activity was detected in all JA-treated clover roots replicates while no TPI activity and therefore no defence induction was detected in untreated control samples (binomial test, P<0.001). In treated roots, inhibition zone diameters were consistently equivalent to those produced by the standard soybean TPI at concentrations between 35 and 70 pM.
7.3.2. *Costelytra* spp. response to increased host defences

After six weeks of feeding on JA-treated clover roots, individuals of both *C. zealandica* populations (exotic pasture and native grassland) had significantly larger biomasses compared to larvae that were fed with untreated roots (Figure 7.1a-b). The treatment effect was significant as early as the beginning of the third week (ANOVA, P values<0.05) (Figure 7.1a-b). On the other hand, no treatment effect was detected for the non-pest *C. brunneum* (ANOVA, P values>0.05 for each week) (Figure 7.1c).

![Figure 7.1](image)

*Figure 7.1.* Average larval weight gain (percentage) for *Costelytra zealandica* (a-b) and *C. brunneum* (c) in response to a diet consisting of clover roots treated with jasmonic acid (black) or untreated controls (grey). Vertical bars represent 5% LSD.

No significant treatment effect was detected with respect to the survival of the larvae of both species (Fisher’s exact test, p>0.05) (Figure 7.2). However, the survival of the pest *C. zealandica* was significantly higher than that of the non-pest *C. brunneum* fed with either JA-treated clover (Fisher’s exact tests, p<0.05) or with the untreated clover (Fisher’s exact tests, p<0.05) (Figure 7.2).
7.4. Discussion

In this study, the direct soil injection of JA resulted in the systemic induction of plant defences in the form of trypsin-inhibitor activity in the root system of *Trifolium repens* (white clover). While above-ground induced immunity is a well understood process which has been characterised in many crop species (for a review see Van der Ent et al. 2009), scant attention has been paid to induced immunity in the below-ground parts of plants (Erb et al. 2012, Pierre et al. 2012). Erb et al. (2012) suggested that induced root resistance might not be as common as induced leaf resistance and proposed that an alternative strategy to JA induced resistance is the release of root volatiles attracting below-ground insect feeders’ natural enemies. This type of strategy implies the existence of substantial co-evolution pattern(s) between the attacker and the attacked species, and is consequently not irrefutable, which suggests that other constitutive and/or inducible defences could also be involved in the insect-plant system studied here. In this respect, the present study showed that *T. repens* has the capacity to increase its below-ground defences through the JA pathway when induced, confirming its suitability for the experiment designed to determine if *C. zealandica* was pre-disposed to overcome the defence of white clover.

Our study showed that, when induced, *T. repens* has the capacity to increase its below-ground defences through the JA pathway and both populations of the pest, *C. zealandica* showed significantly better growth compared with the control treatment when fed with JA-treated white clover roots. In contrast, no treatment effect was observed in the non-pest species *C. brunneum.*
The analyses of the survival rates of *Costelytra* spp. larvae revealed that both species were able to avoid the effect of an increase of their host defences.

The fact that *C. brunneum* showed significantly lower survival rates than its congener *C. zealandica*, both for treated and untreated clover roots, may reflect a certain sensitivity of the species to sampling manipulation, initial health assessment and experimental monitoring. This higher sensitivity in *C. brunneum* was also detectable by the rapid weight increase during the first week of treatment, once the living conditions for the larvae were stabilised. The lower survival rates were not ascribed to *C. brunneum* feeding preferences, which were similar when exposed to their native hosts or to white clover (Chapter 5).

Survival is only one of the many insect fitness traits that can be measured (Orr 2009) which is likely to be affected by increased plant defences. In this study, immature stages of the insects were investigated, so fitness traits such as mating or oviposition performances were not assessed. Increased fitness as measured by higher growth compared with the control was somewhat unexpected for *C. zealandica*, however, recent studies have reported similar responses of insect species to increased plant defences, similarly triggered by JA application or by insect wounding. For example, Pierre et al. (2012) reported that a JA application in turnip plants resulted in an increase of the pupal size of *Delia radicum* (Linnaeus) (Diptera: Anthomyiidae) and Robert et al. (2012) reported that larvae of *Diabrotica virgifera* Le Conte (Coleoptera: Chrysomelidae) gained over 30% more weight on maize plants that were damaged by conspecifics than on healthy ones. The latter study confirms that such fitness increase is not merely an artifact to the JA treatment, but is a genuine response of some insect species to the plant activated immune-system. Although the detailed chemical nature of insect-induced plant defences remains largely unknown (Jansen et al. 2009) and little attention has been given to defence induction in the below-ground parts of plants (Erb et al. 2012, Pierre et al. 2012), it has been established that the central signaling molecule JA triggers a regulatory cascade (Koo & Howe 2009). That cascade is thought to result in the synthesis of numerous primary and secondary plant metabolites (Schwachtje & Baldwin 2008, Jansen et al. 2009). Phenolics have been identified among these final products of defences-related genes mediated by the JA defence pathway (Wolski et al. 2010, Walters 2011), and cases, where their constitutive and inducible occurrence and accumulation in the belowground parts of the plants, have also been reported (e.g. Valette et al. 1998, Wuyts et al. 2007). Phenolics are well known for being detrimental to plant feeding insects and act as antifeedants (Bernays & Woodhead, 1982). Nevertheless, over recent decades, numerous studies have reported counterintuitive positive correlations between high plant phenolics content and high fitness performance of their insect attackers (e.g. Bernays & Woodhead, 1982, Bernays et al. 1983, Kubanek et al. 2004, Jonhson et al. 2011, Sukovata et al. 2011, Pierre et al. 2012). Some studies also suggest that elevated contents of root phenolics rather than being detrimental can have beneficial antioxidant properties
for certain herbivorous insects (e.g. Johnson & Felton 2001, Piskorski et al. 2011). Another example of secondary plant metabolites that may be involved in insect fitness increases are the benzoazinoids. Despite their status of major defensive compounds in maize roots (Robert et al. 2012a, 2012b), these metabolites have been reported to increase the growth of the larvae of *D. virgifera*. Tissues containing high level benzoazinoids have been shown to be preferred by the larvae of this species (Robert et al. 2012b). Nevertheless, in contrast with the previously mentioned phenolics, this last explanation is not tenable for the present study, since clover plants do not produce these secondary metabolites, which solely occur in a restricted group of plants (Hanhineva et al. 2011). Considering that some insects possess the ability to sequester various plant compounds for their own defence (for a review see Jansen et al. 2009); maybe also, some species, such as *C. zealandica* in this study, have the ability of turning some host defences to some sort of fitness advantage. However, another possible alternative explanation may rely on the better use of altered primary root metabolite concentrations such as increases in C/N ratio that have been observed in herbivore-damaged clover roots (Murray et al. 1996) rather than on the direct use of secondary host plant compounds by the insect. In this sense, *C. zealandica* may possess an evolutionary advantage that allows it to cope better with induced host responses than *C. brunneum* and thus allows it to make better use of the high nutrient of these primary metabolites.

While the results of this study suggest that *C. zealandica* may benefit from host defence induction, it is notable that *C. zealandica* collected from isolated native grasslands, never exposed to exotic white clover, also significantly increased weight when fed with the JA-induced ‘new’ host compared with the insects in control treatments. Such a result suggests that the ability to avoid the detrimental effects of plant defences and seemingly benefit from them, has not resulted from an “arms race” between the insect and its host, but is a pre-adaptation. The ability to avoid detrimental effects of host defence chemicals and to benefit from them has been consistently reported in particular reference to invasive species. For example, Sukovata et al. (2011) reported that high concentrations of total plant phenols were favorable to grub development in the common cockchafer, *Melolontha melolontha* (Linnaeus) (Coleoptera: Scarabaeidae), another notorious pest (Enkerli et al. 2008) from the Melolonthinae subfamily. Similar observations have been reported in other coleopteran pests, such as in the vine weevil *Otiorhynchus sulcatus* Fabricius (Coleoptera: Curculionidae) (Johnson et al. 2011) and in the western corn rootworm *D. virgifera* (Erb et al. 2012). Such observations are not restricted to coleopteran species, and have been reported in invasive moths and grasshoppers (Bernays & Woodhead 1982, Bernays et al. 1983) and more recently for an invasive marine species of crustacean (Kubanek et al. 2004).

Despite previous reports, to our knowledge, this study is the first to hypothesise a link between insect invasion success and a pre-existing ability to overcome and benefit from the defence
metabolites of a new host. Such a key trait could have important implications for improving pest risk assessment to prevent and predict the potential economic impact of newly introduced phytophagous insects into new areas.
Investigation of the relationship between the PGI enzyme system in two Costelytra species and their fitness response to temperature as measure of environmental tolerance

Abstract

In the field of invasion ecology of insects, the determination of a species environmental tolerance, particularly thermal tolerance, is essential to predict its potential distribution. Through its effects on metabolic rate, temperature is often considered the most important abiotic factor that affects numerous insect life-history and fitness traits. Investigating the effect of challenging temperatures on a selected organism can provide key information to support spatial and climatic distribution as well as predictive studies, particularly under the current context of global warming. Therefore, the study of the metabolic enzyme-system phosphoglucose-6-isomerase (PGI), in invasive species and under various temperature regimes, may be informative. The present chapter aimed to, (1) compare the thermal tolerance of an invasive species to that of a non-invasive congener, and, (2) investigate whether the PGI enzyme system, which has been frequently correlated to various insect life-history traits, could be a key enzyme in the invasion success of some insects. Using the invasive pest scarab, Costelytra zealandica (White) and its close congenic non-invasive species, C. brunnneum (Broun), it was investigated whether particular PGI genotypes were related to better fitness performance under different temperature regimes. Third instar larvae of each species were exposed to three different temperatures (i.e. 10, 15 and 20°C) over six weeks and their fitness (survival and growth rate) measured. No relationship was detected between PGI genotype and fitness in this particular model species, suggesting that the PGI may not be related to the invasion success of C. zealandica despite its recognised status as a key enzyme in insect thermal adaptation.

8.1. Introduction

In invasion ecology, measuring the environmental tolerance of insects, particularly thermal tolerance, is crucial to predict their potential geographic and ecological range. Temperature is well known to limit insect development and therefore their geographical range and to influence their population dynamics (Wallner 1987, Clarke 2003, Sinclair et al. 2012). Because of its effects on metabolic rate, temperature is considered the most important abiotic factor directly affecting
phytophagous insects (Bale et al. 2002) through its direct effect on a number of life-history and fitness traits (Clarke 2003, Karl et al. 2008), among which growth and survival (Folguera et al. 2010) are the most important. For instance, McMillan et al. (2005) reported a significant increase in larval mortality of the leaf beetle *Chrysomela aeneicollis* Schaeffer in the coldest river drainage of the three tested in this study, which was exposed to subzero night-time air temperature. In another study involving the larvae of the Glanville fritillary butterfly, Kallioniemi and Hanski (2011) reported similar low survival rates under stressful conditions represented by a low-temperature treatment. Similarly, but with regard to high temperatures, Papanikolaou et al. (2013) showed that the development of the immature stages of the 14-spotted ladybird beetle could be greatly impaired by challenging temperatures. They reported that the highest mortality rates in the immature stages of the 14-spotted ladybird beetle occurred under the two highest temperatures tested in this study. Others studies have suggested that invasive species may have a broader and greater physiological tolerance over a wide range of temperatures than native species sharing the same thermal habitat (for a review see Zerebecki & Sorte 2011). Therefore, investigating the effect of challenging or extreme temperatures on threatening insect species can provide key information to support spatial and climatic distribution projections for a range of risk assessments particularly under the current context of global warming.

The extended geographical occurrence and recognized negative impact on most New Zealand agro-ecosystems of *C. zealandica* suggests that it has reached a very high degree of invasiveness within its home range. In fact, based on early observations, this native insect seems to have become so widespread that it is only absent from a few remote locations of New Zealand (Given 1966) and has not reached the status of invader only in few others (East et al. 1981). The widespread distribution of *C. zealandica* is likely to be in part related to its tolerance to a wide range of soil temperature, within the array of those encountered throughout New Zealand (Section 5.4). Recently, Sinclair (2012) stated that numerous insect species maximise their performances under varying conditions because of a high degree of phenotypic plasticity. For example, Craig Stillwell and Fox (2009) demonstrated that different temperatures significantly impact survivorship, body size and fecundity in the seed beetle *Stator limbatus* (Horn). They concluded that the differential responses relied on a high degree of phenotypic plasticity rather than on a genetic adaptation resulting from long-term evolution. Similarly, the initial spread of *C. zealandica* within its native range not only would have relied on the widespread cultivation of its new host and suitability of soil temperatures accessible within its range, but maybe also on the ability of this species to adjust to variation of this environmental factor. The spread of *C. zealandica* greatly contrasts with the restricted range of the non-pest species *C. brunneum* that remains confined to few patchy areas throughout New Zealand. Based on the examination of several museum insect collections (personal observation, see Section 2.2.1), the distribution of *C. brunneum* appears to be mostly located in the New Zealand Southern Alps. These differences in the distribution of *C. zealandica* and *C. brunneum* seem to corroborate the observation made by
Zerebecki & Sorte (2011), who empirically studied temperature tolerance and stress proteins in invasive species and conclude that these species tend to live within broader habitat temperature ranges and higher maximum temperatures.

Several enzyme systems have been either successfully linked or are suspected to play a key role in animal physiological tolerance to temperature. For instance, lactate dehydrogenase-B (LDH-B) has been linked to thermal tolerance in a killifish species (Johns & Somero 2004, Dalziel et al. 2011). The adapting kinetic properties of the cytosolic malate dehydrogenase (cMDH) enzyme were related to warm temperature adaptation in blue mussels (Fields et al. 2006), and similarly, the locus Idh-1 of the enzyme system isocitrate dehydrogenase has been shown to exhibit significant correlations between allele frequencies and temperature in several species (for a review see Huestis et al. 2009). For invasive species, Hanski and Saccheri (2006) also suggested that the metabolic enzyme system phosphoglucone-6-isomerase (PGI) could play a key role in the range expansion and delineation of the geographical range boundaries of these species. This enzyme system sits at the intersection of the major glycolysis and glycogen biosynthesis metabolic pathways, catalyzing the second step in glycolysis to energy in the form of ATP to the organism (Riddock 1993). Because of this unique biochemical situation, it is considered that particular covariation patterns between individual fitness performance or life-history traits and Pgi genotypes are likely to arise (De Block & Stock 2012).

The phenotypic variability of the PGI enzyme system has been correlated many times to insect fitness performance. For instance, several recent studies on the Glanville fritillary butterfly have examined the possibility of a link between allozyme variations at this enzyme locus and insect life-history and fitness traits, characters which might also be relevant to success of an invasive species. Haag et al. (2005) established that genetic variation in Pgi locus was correlated with flight metabolism, dispersal rate and metapopulation dynamics in this butterfly. Additionally, Hanski and Saccheri (2006) showed that the allelic composition of the PGI enzyme system had also a significant effect on the growth of local populations of the same species. The link between long lifespan duration and PGI genotype showing high dispersal capacity was also demonstrated (Saastamoinen et al. 2009). But even more relevant to the present study, PGI has been designated several times as a key enzyme candidate in insect thermal tolerance to extreme temperatures (for a review see Kallioniemi & Hanski 2011), and as such, has been characterised as the best-studied metabolic enzyme in a recent review about variation in thermal performance in insect populations (Sinclair 2012). Despite this, it seems that this enzyme system has never been analysed in a comparative study involving invasive/pest versus non-invasive insect species.

As part of an investigation to help explain invasiveness in C. zealandica the first aim in this chapter was to investigate the hypothesis that a phytophagous insect that becomes a successful invader on a new host in its native range is more tolerant of a wider range of temperature, that
facilitates establishment over wider geographic areas in contrast with a closely related non-pest species. The second aim was to establish whether particular PGI-genotypes are related to individual fitness advantage in the model species *C. zealandica* and *C. brunneum* when exposed to realistically challenging temperatures as defined in Chapter 5.

8.2. Material and methods

8.2.1. Insect sampling

One population of *C. zealandica* was collected in the South Island of New Zealand (sample site B) while another was collected from the North Island (sample site E) (Section 2.2.1). To limit the number of variables in this study, the sample sites selected for *C. zealandica* were both exotic pastoral sites (section 2.2.1), so host-associations were similar for the two populations of *C. zealandica* studied. For comparison, one population of the non-invasive species *C. brunneum* was sampled from site D (section 2.2.1). Third instar larvae were collected at each site and identified to species level based on the methodology described in Chapters 3 and 4 and in Lefort *et al.* (2013). Prior to experimentation, all larvae were tested for amber disease (Section 2.2.3.1) and only healthy larvae were used.

8.2.2. Survival and growth response of two *Costelytra* species to different temperature regimes

*Costelytra* larvae usually live at an average soil depth of 10 cm (Wright 1989). At this depth, and because of the resulting buffer effect, the yearly maximum temperatures rarely reach 20°C and often remain above 5°C during the coolest months of the year in New Zealand (see Chapter 5, Figure 5.1). Because of the univoltine nature of the *Costelytra* species life-cycle pattern (see Chapter 2, Figure 2.2), feeding third instar larvae are rarely exposed to soil temperatures below 10°C for long periods. Therefore 10 and 20°C were used as relatively challenging temperatures within the normal range of these species, while 15°C, the standard rearing temperature (Section 2.2.3.2), was used as control.

The larvae of each population (n = 90 for each *C. zealandica* population, and n = 30 for *C. brunneum* population) were randomly allocated to one of the three temperature treatments and each larva reared individually as described in Chapter 2, but under the particular incubator temperatures used in this study.
All larvae were fed *ad libitum* with chopped roots of *Trifolium repens* (white clover) plants grown following the methodology described in Chapter 2 (Section 2.2.3).

Larval survival and growth measured as weight gain were recorded as measures of fitness, and assessed weekly over a period of six weeks. Dead larvae were collected every 24 hours, placed in 2 ml plastic vials and stored at -80°C. At the end of the experiment, all the larvae were snap frozen and stored in similar plastic vials at -80°C, to avoid any protein degradation, until processed in an electrophoretic study.

Statistical analyses to determine the effect of temperature on larval survival were carried out using Fisher’s exact tests. Growth data were analysed by analysis of variance (one way ANOVA) after exclusion of larvae that died before the end of the six weeks experimental period. Statistical tests were conducted with R software (R Development Core Team 2009) and GenStat® (GenStat 14, VSN International Ltd, UK).

### 8.2.3. PGI electrophoretic study

The last abdominal segment of each larva (Section 8.2.2) was cut into small pieces on a square glass plate over ice and subsequently transferred into 1.7 ml cooled Eppendorf microtubes. The abdominal segments were then ground, using an autoclaved plastic rod, in 100 µl of cooled extraction buffer (Tris-HCl, pH 8.0), until complete homogenisation.

After determination of optimal electrophoresis parameters for the enzyme system and the species studied (Appendix 3 – Cellulose acetate electrophoresis optimisation, general advices and example of the PGI enzyme system in *Costelytra* species), the PGI allozyme expression of each of these larval extracts was subsequently examined by electrophoretic analysis following the Hebert and Beaton (1993) methodology. The final procedure comprised loading 10 µl of each homogenate on the well of a sample plate (Super Z-12 applicator kit, Helena Laboratories). Immediately after, a cellulose acetate plate (Titan® III 76 mm x 76 mm cellulose acetate plate, Helena Laboratories), which had been left to soak in 100 ml of electrode buffer (Table 1) for 30 min, was blotted dry using filter paper and placed, mylar side down, on an aligning plate (Super Z-12 applicator kit, Helena Laboratories). Using an extract applicator (Super Z-12 applicator kit, Helena Laboratories), a series of 10 extracts, plus one positive heterozygote control for each *Costelytra* species, was collected from the wells of the sample plate and applied to the cellulose acetate plate. Once loaded, the plate was placed, acetate face down, on buffer soaked wicks over the partition of the electrophoresis tank (Figure 8.1) filled out with cooled Tris-Glycine electrode buffer pH 8.5 (Table 8.1).
Figure 8.1. Electrophoresis tank set up. (1) Electrode buffer (2) Sample loading zone (3) Cellulose acetate plate (4) Tank partition (5) Wick. Adapted from Hebert and Beaton (1993).

The electrophoresis was carried out at a constant voltage of 200 V and 2 mA for 15 min. After the completion of the electrophoresis, the plate was removed from the tank and immediately stained with 4 ml of a freshly prepared PGI-specific stain mix (Table 8.1).

Table 8.1. Stock solution recipes and their use for cellulose acetate electrophoresis targeting the PGI enzyme system. Based on Hebert and Beaton (1993)

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Final products</th>
<th>Use(s)</th>
<th>Recipe-Chemicals</th>
<th>Quantities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrode buffer</td>
<td>Tris-Glycine (x10)</td>
<td>(1) plate soaking (2) fill up electrophoretic tank (3) wicks soaking</td>
<td>Trizma base Glycine Distilled water</td>
<td>30 g 144 g make up to 1 litre</td>
</tr>
<tr>
<td>Gel buffer</td>
<td>Tris-HCl (0.09M, pH8)</td>
<td>(1) stain recipe chemical component</td>
<td>Trizma base HCl (1M) Distilled water</td>
<td>44.4 g 248 ml make up to 4 litres</td>
</tr>
<tr>
<td>Stain</td>
<td>PGI-specific stain mix</td>
<td>(1) plate staining</td>
<td>Tris-HCl (0.09M, pH8) NAD Fructose-6-phosphate MTT PMS G6PDH Agar</td>
<td>1 ml 1.5 ml 5 drops 5 drops 5 drops 10 μl 2 ml</td>
</tr>
</tbody>
</table>

The agar-based staining overlay was removed by holding the plate under cold running water. Staining time was estimated visually and lasted between 1 and 2.5 minutes to resolve the enzyme. The plate was then soaked for 30 minutes in water, blotted dry and placed in an oven at
60°C for 15 minutes. Finally, all plates in the experiment were digitised using a UVIDOC HD2 (Uvitec Cambridge, UK) and band scoring was performed by optimising the definition and aligning the different allozyme profiles obtained using Adobe photoshop CS5 and OmniGraffle 5 Professional (Figure 8.2). For each population studied, heterozygote and homozygote forms were scored for each population studied, and homozygotes forms were distinguished by allele assignment as slow or fast based on their relative mobility from the loading zone (Figure 8.2c).

**Figure 8.2.** Example of scoring of a cellulose acetate plate. Where (a) is an original picture of the plate, (b) is a definition optimisation and alignment of the allozyme profiles via Adobe Photoshop CS5 and OmniGraffle 5 Professional and (c) is a representation of the allozyme profiles. H1, fast allele detected - homozygote 1; H2, slow allele detected - homozygote 2; HE, fast and slow allele detected – heterozygote; SLD, sample loading zone.

Comparisons to determine differences of larval survival rates in relation to populations, allozyme(s) form expressed and temperature regimes, were carried out using Fisher’s exact tests (Figure 8.3).

The PGI-genotype obtained for each larva (i.e. homozygote vs heterozygote) was tested for any correlation with fitness response (i.e. larval survival and total growth) within the different temperature regimes. The effect of each treatment on larval growth was analysed using analysis of co-variance (ANCOVA), with the genotype of each larva used as a co-variate and where homozygotic genotypes were assigned 1 and heterozygotes 0. The analysis was performed after exclusion of larvae that died before the end of the six weeks experimental period and therefore for which total growth data was not available. All growth measurements were transformed to percentage weight gain with respect to the initial weight of the larvae, prior to the analysis.
8.3. Results

8.3.1. PGI electrophoretic study

The electrophoretic study revealed the existence of only one PGI-locus in *Costelytra* species (Figure 8.2). The genotypes of the two species, along with their distribution in each temperature treatment are summarized in Table 8.2.

Table 8.2. PGI genotypes detected by cellulose acetate electrophoresis in *Costelytra zealandica* and *C. brunneum* and their effective distribution in each temperature treatment.

<table>
<thead>
<tr>
<th></th>
<th>H1 (homozygote - fast allele)</th>
<th>H2 (homozygote - slow allele)</th>
<th>HE (heterozygote)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. zealandica</em> (sampling site B)</td>
<td>n=14 (respectively n= 8, 3 and 3 at 10, 15 and 20°C)</td>
<td>n=16 (respectively n= 7, 2 and 7 at 10, 15 and 20°C)</td>
<td>n=36 (respectively n= 9, 14 and 13 at 10, 15 and 20°C)</td>
</tr>
<tr>
<td><em>C. zealandica</em> (sampling site E)</td>
<td>n=13 (respectively n= 5, 3 and 5 at 10, 15 and 20°C)</td>
<td>n=43 (respectively n= 17, 15 and 11 at 10, 15 and 20°C)</td>
<td>n=23 (respectively n= 8, 9 and 6 at 10, 15 and 20°C)</td>
</tr>
<tr>
<td><em>C. brunneum</em> (sampling site D)</td>
<td>n=10 (respectively n= 1, 5 and 4 at 10, 15 and 20°C)</td>
<td>n=4 (respectively n= 1, 2 and 1 at 10, 15 and 20°C)</td>
<td>n=11 (respectively n= 6, 2 and 3 at 10, 15 and 20°C)</td>
</tr>
</tbody>
</table>

8.3.2. Effect of the different temperature regimes on larval growth and survival

Over the temperature treatments applied to the three populations, the response to only two temperatures for *C. zealandica* collected from the North Island of New Zealand (sample site E) were significantly different. For this population, 100% of the larvae survived at the lowest temperature of 10°C which was significantly different from the 77% survival rate of the larvae at 20°C (Figure 8.3).
Figure 8.3. Percentage of larval survival of two populations of *Costelytra zealandica* and one population of *C. brunneum* after 6 weeks of treatment under different temperatures (10, 15 and 20°C) and details of larval survival (dark grey)/mortality rates (light grey) observed for each PGI-genotype detected in each population. Total sample sizes were n=90 for the two *C. zealandica* populations and n=30 for *C. brunneum*.

However, the weight gain of the North Island larvae (i.e. sample site E) that survived the 6 weeks of experiment was not significantly different at any temperature (Figure 8.4).
Figure 8.4. Average weight gains of the surviving larvae of two populations of *Costelytra zealandica* and one population of *C. brunneum* after 6 weeks of treatment under different temperatures (10, 15 and 20°C). Vertical bars represent 5% LSD.

In contrast with the North Island population, the weight gain of the South Island larvae (*C. zealandica* from the sample site B) significantly increased under the highest temperature of 20°C compared to the lowest temperature tested (Figure 8.4). In contrast with *C. zealandica*, the non-pest species *C. brunneum* gained, on average, significantly more weight at the selected average temperature of 15°C (Figure 8.4).

### 8.3.3. Temperature tolerance & PGI-genotypes

The results of the ANCOVA revealed that there was no significant relationship of the PGI-genotypes of the larvae with their total weight gain under various temperature regimes (Table 8.3). Nonetheless, the ANCOVA demonstrated that there was a marginally significant effect of the different temperature regimes on larval growth of the three populations tested at 10% level of significance (Table 8.3).
Table 8.3. ANCOVA results for the effects of PGI-genotypes on Costelytra zealandica and C. brunneum larval growth (given in bold), where the main treatment was the temperature (i.e. 10, 15 or 20°C) and where the genotype of each larva is used as co-variate and where homozygotes, whatever the allele form expressed, were assigned number 1 and heterozygotes number 0.

<table>
<thead>
<tr>
<th>Species (sampling site)</th>
<th>df</th>
<th>F</th>
<th>P values</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. zealandica (site B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>2.42</td>
<td>0.098</td>
<td>marginally ‡</td>
</tr>
<tr>
<td>Co-variate</td>
<td>1</td>
<td>0.29</td>
<td>0.589</td>
<td>ns</td>
</tr>
<tr>
<td>C. zealandica (site E)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>2.76</td>
<td>0.069</td>
<td>marginally ‡</td>
</tr>
<tr>
<td>Co-variate</td>
<td>1</td>
<td>0.05</td>
<td>0.830</td>
<td>ns</td>
</tr>
<tr>
<td>C. brunneum (site D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>2.74</td>
<td>0.087</td>
<td>marginally ‡</td>
</tr>
<tr>
<td>Co-variate</td>
<td>1</td>
<td>0.31</td>
<td>0.586</td>
<td>ns</td>
</tr>
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</table>

8.4. Discussion

The main objective of this chapter was to investigate the suggestion proposed by Hanski and Saccheri (2006) that the Pgi gene may be strongly implicated in ‘the expanding front of invasive species’. The present chapter aimed at testing whether a relationship between the PGI-genotypes of Costelytra species and their fitness response under different temperature regimes exists. The results of the experiments undertaken in this study did not detect any such correlation, although significant effects of the various temperature regimes tested were detected on the larval growth and/or survival of the two species studied.

As shown in Chapter 5, C. zealandica survived better at lower rather than at higher temperatures, however in the present experiment only the population collected from the North Island of New Zealand showed significantly better survival rates at 10°C compared with the highest temperature tested of 20°C. The fact that no significant temperature effect was detected on larval survival for the population collected from the South Island could be due to the shorter length of this experiment compared to the one performed in Chapter 5 which lasted for 17 weeks. Additionally,
the larvae collected from the North Island could have been more sensitive to the experimental conditions, because they were subject to a higher degree of disturbance and stress associated with a longer transport from the sample site to the laboratory (Section 2.2.1). The delays may have caused adverse effects on some aspect of the health of these larvae that may have interacted with the results of the subsequent experiment, such as indicated by a greater number of larvae that died under the more challenging temperature(s) (i.e. 10 and/or 20°C). In a similar way, although the trends at a first glance appeared similar, the temperature effect on larval growth was significantly different between the two populations of *C. zealandica* investigated. As shown in Chapter 5 the weight gain of the larvae collected from the South Island was significantly depressed by low temperature (i.e. 10°C), whereas this effect was not detected in the larvae collected from the North Island. Here, some degree of genetic divergence between the two populations studied might exist and explain this disparity. For example, the cosmopolitan *Drosophila melanogaster* Meigen exhibits complex patterns of genetic variation between populations that allowed it to successfully establish worldwide under extremely diverse thermal environments (Hoffmann et al. 2003, Morgan & Mackay 2006). Based on the comparison of ITS1 rDNA sequences of several populations of *C. zealandica*, some degree of genetic divergence within this species was reported by Richards et al. (1997), particularly between North Island and South Island populations. Furthermore, adult specimens of *C. zealandica* have been reported to be larger in the North Island (Travis Glare, personal communication, Bio-Protection Research Centre NZ). In addition, and as discussed in Chapter 6, host-race formation in this species may have occurred. This may have contributed to the establishment of even further genetic divergence between *C. zealandica* populations throughout New Zealand compared with those reported by Richards et al. (1997), although in the present study, both populations were collected from exotic pastures mostly composed of the same host plants (Table 2.2). The non-pest species *C. brunneum*, in contrast to *C. zealandica*, showed significantly impaired larval growth under the most challenging temperature regimes, particularly at 20°C. These challenging temperatures were however not lethally detrimental for *C. brunneum*. Even though very little is known about its actual distribution, *C. brunneum* seems to prefer mid to high altitudinal ranges (Hoy & Given 1952, Given 1966), where soil temperatures are likely be similar to the averages recorded in the coldest southern locations of New Zealand (e.g. Invermay’s yearly average soil temperature ranged between 15 and 2.9°C, see Figure 5.1 in Chapter 5). These observations, corroborate the working hypothesis of this thesis that this species is less tolerant than *C. zealandica* to challenging temperatures, particularly with regard to high temperatures, and failed to further extend its geographical range unlike its congener.

Because of its potential impact on the expression of genes encoding for metabolic enzymes and on the functional properties of these enzymes (Kallioniemi & Hanski 2012), temperature is often characterised as a key environmental factor affecting the growth and survival of poikilo thermal organisms (Kallioniemi & Hanski 2012, Sinclair et al. 2012). Hence, the interest in the expression
of various forms of metabolic enzymes, so called allozymes or isoenzymes, has considerably increased over recent decades (Karl et al. 2010). The PGI has been described as a highly polymorphic enzyme system in numerous taxa (Kallioniemi & Hanski 2012). For instance, seven alleles were detected for the pgi gene in Melitaea cinxia (Linnaeus), the Granville Fritillary butterfly, and many coleopteran species possess over three alleles for this gene (e.g. Nährung & Allen 2003, Dahlhoff & Rank 2007). In the present study, the electrophoretic profiles for the two species of Costelytra studied were interpreted based on the relative mobility of alleles with regard to the loading zone and were interpreted as the expression of two alleles at only one PGI-locus (Figure 8.2). However and because of the quite unusual net charge of the various PGI allozymes synthesised by both Costelytra species, which migrate toward the cathodal electrode (Appendix 3) resulting in relatively short migration of the proteins from the loading zone, it is possible that misinterpretation of the gel has occurred. Such a possibility leads to the consideration that maybe the detection of additional alleles was missed because of poor migration and separation of the various allozymes on the gel. Indeed, protein mobility in an electric field depends upon its net charge that is dictated by the side chains of amino-acid, which can have an acidic or a basic group (i.e. COO- or NH4+), and also by its amino-acid sequence of the polypeptide chain, which is responsible for the net surface charge and the shape of the protein (Richardson et al. 1986). Although quite unlikely, the differences in the amino-acid sequence of two allozymes of the same gene may be such so they have a similar—or highly similar—net charge. Therefore such possibility must be considered, and failure to detect additional alleles would explain why this enzyme system appeared not quite as polymorphic as it does in other insect species. High pH electrode buffers are usually considered to provide a better separation of the various allozymes in such situation (Hellena Laboratories 1976, Richardson et al. 1986). While the use of different buffer systems did not improve the resolution of the gel in this study (Appendix 3), the use of another support medium such as starch or polyacrylamide gels would possibly provide better migration patterns for the PGI enzyme system in Costelytra species. Alternatively, the use of mass spectrophotometry could help to establish whether or not additional allozymes are expressed in these species.

Extensive studies on PGI expression in several butterfly species have demonstrated that enhanced individual performances can be given by different allelic variations depending on the life-history traits investigated. For instance, Karl et al. (2008, 2009) demonstrated that there was enhanced larval and pupal growth and development in heterozygote genotypes PGI 2-3 in the butterfly Lycaena tityrus, whereas cold stress resistance, in the same species, was associated with a different PGI genotype. Therefore, if the low allelic variability observed for Costelytra species in this study is the result of a mis-interpretation of the electrophoresis profiles, it could explain why no relationship between individual larval fitness responses and PGI genotypes were detected. On the other hand, if the interpretation of electrophoresis profiles is accurate, and if as hypothesized the PGI enzyme system was linked to individual tolerance to challenging
temperatures in *Costelytra* species, two alleles would have been enough to detect it. Putatively, heterozygote individuals for this enzyme system should have displayed better weight gain and survival rates under challenging temperatures, presumably because their PGI-associated metabolic pathways would have been able to adapt better to a wider range of temperatures than that of homozygotes. Such results would be consistent with Watt’s studies (1977, 2003) of the PGI enzyme system in *Colias* butterflies, which reported heterozygotic advantage with respect to several life-history and fitness traits in this species. Additionally, the fact that no relationship was detected between the selected life-history traits and the PGI genotypes of the species studied may also be due to the experimental design that resulted in small and unbalanced sample sizes as shown in Table 8.2.

Although the present study did not support the possibility of a relationship between the PGI enzyme system and the fitness response of *Costelytra* species to temperature, and because relationships between PGI genotype and thermal tolerance have already been detected in other insect species, further investigations using other species models and other experimental designs would certainly be worthwhile. These are further addressed in the general discussion of this thesis (Chapter 9), particularly the possibility of investigating heat shock proteins expression rather than the PGI enzyme system as has been done in a study performed on a leaf beetle by Dalhlhoff and Rank (2000, 2007).
Chapter 9

General discussion and concluding remarks

9.1. Introduction

Hierro et al. (2005) have demonstrated the importance of undertaking comparative studies of exotic invaders in both their introduced and native home ranges. However, as established in Chapter 1, some cases exist where such comparative studies cannot be conducted, simply because the invaded and the home ranges of the species in question cannot be distinguished geographically. In such cases, particularly when studying phytophagous insects, the use of populations which either feed on ancestral or novel host plants in their native range, can serve as a useful alternative to perform comparative studies. Furthermore, and as suggested in Chapters 1 and 5, the comparison of a native invasive species with a native but non-invasive congener has potential to provide informative data for studies attempting to identify characteristics that promote invasiveness in native species. Most theories and explanations invoked to explain invasion success have been developed based on the study of exotic species or species already established in their exotic invaded range and therefore may not be as tenable for native species (Section 1.3.4). Therefore, the present thesis aimed to investigate some mechanisms that may help explain why some phytophagous insect species become invasive in their native range, by using several populations of C. zealandica, the New Zealand native and invasive scarab, and C. brunneum a close non-invasive congener, as model species.

Although bottom-up and top-down theories, such as the enemies release hypothesis (ERH) (Elton 1958) or the novel weapon hypothesis (NWH) (Callaway & Ridenour 2004), are often mentioned to explain exotic species invasion (Table 1.1), the present thesis focused on the investigation of the intrinsic characteristics of the invader itself rather than on external factors that might have contributed to its success. This decision was made based on the assumption that external factors influencing the success of native and an exotic invaders are likely to differ because of the characteristics of their ‘new habitat’ (Section 1.3.2) while, in contrast, intrinsic key traits supporting invasiveness are more likely to be similar in both exotic and native species. Therefore, the research in this thesis was expected to offer new insight regarding the invasive potential of phytophagous insect species in general.
The three main hypotheses of this thesis were:

Hypothesis #1. A phytophagous insect that becomes a successful invader on a new host in its native range has higher fitness on that host compared with its ancestral host, in contrast with a closely related non-pest species.

Hypothesis #2. A phytophagous insect that has higher fitness on a new host species is better able to overcome the defences of that new host compared with a closely related non-pest species.

Hypothesis #3. A phytophagous insect that becomes a successful invader on a new host in its native range is more tolerant of a wider range of environmental conditions, particularly with regard to temperature, in contrast to a closely related non-pest species.

The six main objectives initially established to address the above research hypotheses (Section 1.6) were all addressed by one or several chapters and are summarised and discussed below within context of other published research. Chapters 2, 3 and 4 concentrate on methodological challenges presented by the use of scarab larvae and cryptic species as the subjects of this research (Objective 1), while Chapters 5, 6, 7 and 8 focus on the investigation of intrinsic key traits associated with the hypotheses mentioned above (Objectives 2 to 6).

9.2. Morphological crypticity and insect larvae

A well-known case of cryptic species complex discovery is that described by Hebert et al. (2004), where a single butterfly originally described in 1775, was established as a complex of at least 10 species. Since that discovery, the use of DNA barcoding techniques has been extensively employed to unravel similar hidden species complexes in various taxa (e.g. Collins 2012, Boyer et al. 2012). One recent example among the realm of insects was reported by Dinca et al. (2011) who established that the common wood white butterfly *Leptidea sinapis* (Linnaeus) consisted of three distinct species, based on the examination of the mitochondrial marker COI and other mitochondrial and nuclear genes. Recent trends in DNA barcoding research have been to determine optimal analytical methods for species delimitation (e.g. Boykin et al. 2012, Brown et al. 2012, Collins et al. 2012). And more recently, Svensson et al. (2013) also proposed a new method based on the combination of DNA barcoding and the use of pheromone trapping to help to resolve cryptic species complexes. By combining these two techniques, Svensson et al. (2013) showed that two populations of the spruce seed moth, *Cydia strobilella* Linnaeus, which expressed distinct sex pheromones, were in fact two different species. As in the previous
examples, the study of crypticity appears to focus most often on the adult form of the insect. In this thesis however, it was the immature-life stages of *C. zealandica* and *C. brunneum* that were investigated. Because the adult stage of the model species chosen for this thesis are morphologically distinguishable (Given 1952, Given 1966, Chapter 3) and have never been erroneously confused when described, these two species cannot be qualified as ‘true cryptic species’ according to the definition by Bickford et al. (2007). Nonetheless, the immature life-stages of *C. zealandica* and *C. brunneum*, have homogenous morphology typical of larvae of scarab beetles (Bain 1980, Capinera 2008) and have remained impossible to tell apart until the research carried out in this thesis (Lefort et al. 2012, Lefort et al. 2013, Chapter 3 and 4). Therefore, the larval stages of *Costelytra* investigated were considered to qualify as cryptic in the present thesis. This could however be perceived as a misuse of terminology, as similarly discussed for the term “invasive” in Chapter 1. Hence, the term ‘pseudocryptic’ as defined by Collins (2012) based on remarks by Smith et al. (2007), could apply more accurately to *Costelytra* larvae, because morphological differences between the congener of the two species became apparent once further taxonomic assessments were performed (Lefort et al. 2013, Chapter 4). Indeed, this thesis established that as many as three characters, out of the four investigated, were morphologically different between the larvae of *C. zealandica* and *C. brunneum* (Lefort et al. 2013, Chapter 4). Furthermore, the overall shape of the septula of the raster, was enough for preliminary differentiation of the two species in the field and, for ambiguous cases, the calculation of the ratio of the length by width of the septula allowed definitive species identification (Figure 4.4.).

The challenges imposed by cryptic species and/or cryptic life-stages are numerous and well acknowledged. With regard to invasive species, Boykin et al. (2012) have raised the issues related to crypticity and biosecurity, and other studies have highlighted the importance of correct species identification for the accomplishment of successful biological control (Rosen 1986, Paterson 1991, Silva-Brandão et al. 2013). In addition, the research reported in this thesis, points out the importance of the detection of immature life-stage crypticity for pest control management and strategies (Lefort et al. 2012, Lefort et al. 2013, Chapters 3 and 4). In New Zealand, in addition to *C. brunneum*, *C. zealandica* co-occurs with nine other congeneric species (Given 1952, 1960, 1966). Among those species, *C. diurna* was first reported in ‘droves of thousands’ in Canterbury (Given 1966) but was only detected once a few years later (Richards et al. 1997), and was thereafter never mentioned again in the literature. Whether such sparse records suggest initial misidentification, crypticity, or even the possibility of becoming rare or extinct, the possibility of *C. diurna* or other species belonging to this genus that co-occur sympatrically with *C. zealandica* and which similarly exploit exotic host plants must be considered. Therefore, care must be taken during species sampling and identification for research purposes, because very similar morphological larvae to that of *C. zealandica* and *C. brunneum* may exist. Based on the known geographical occurrence of the species belonging to the genus *Costelytra* (Given 1952,
1960, 1966, Chapter 2), and potential presence of cryptic larval forms along with C. zealandica, species identification based on DNA barcoding should be preferred. The present thesis offers such alternatives, where two new molecular methods for the identification of larvae of closely related scarab species, have been developed (Chapter 3). Both methods were based on the amplification of the mitochondrial gene COI using larval frass and larval exuviae (Figure 3.2). Additionally the methods were designed to be non-invasive and therefore compatible with any type of behavioural, developmental or fitness studies (Lefort et al. 2012, Chapter 3).

9.3. Phenotypic plasticity and invasion success in native phytophagous insects

Phenotypic plasticity, as defined as the ability of an organism to change and adapt in response to changes in its environment (Price et al. 2003), is often investigated in ecological and evolutionary studies and described as a fundamental component of speciation and evolution patterns (Gorur 2000). Additionally, in recent studies, phenotypic plasticity has sometimes been mentioned to characterise invasiveness in exotic species (Table 1.1, e.g. Duncan et al. 2003, Dzialowski et al. 2003, Chown et al. 2007), although the idea that high phenotypic plasticity could contribute to the success of invasive species was first proposed a long time ago (Baker 1965). The work presented in this present thesis indicates that phenotypic plasticity could be a fundamental component of the invasion success in native phytophagous insects, by demonstrating that the invasion success of C. zealandica most likely depends upon its capacity to quickly adjust to environmental variation or change (Chapters 5, 6, 7, and 8) without the requirement of significant evolutionary adaptation. Similar findings, although based on exotic invasive plant species, were recently reported by Davidson et al. (2011). Their study, which was based on the meta-analysis of 75 invasive and non-invasive species pairs, was carried out to determine the role of phenotypic plasticity in plant invasion success. The results of their research revealed that exotic invasive plant species demonstrate significantly higher phenotypic plasticity than non-invasive species with respect to several fitness measures, for example, biomass, root to shoot ratio and another four additional traits out of nine others investigated (Davidson et al 2011).

When considering species in their native range, their environment may either be affected by, (1) relatively rapid and sudden changes such as the introduction of a new host-plant, (2) repetitive and predictable changes such as annual changes in temperature regimes, or (3) gradual and long-term changes such as global climate change. As suggested by Whitman and Agrawal (2009), such diversity of environmental change could mean that phenotypic responses, whether adaptive or not (Via et al. 1995), may be just as diverse. Richards et al. (2006) defined three types of possible phenotypic responses related to fitness traits, especially with regard to plant
species and their environment. The first response called ‘Jack-of-all-trades’, describes a scenario whereby plasticity allows an invasive species to maintain constant fitness across many different environments, in contrast with a non-invasive species that performs poorly in some of these environments. The second response, called ‘Master-of-some’, in contrast with the ‘Jack-of-all-trades’ scenario, means that the success of an invasive species relies on its ability to rapidly take advantage of newly available resources. In this second scenario, the invasive species shows greater fitness response to favorable conditions than a non-invasive one. Finally, the third response described by Richards et al. (2006) corresponds to a combination of the two previous ones and is qualified as a ‘Jack-and-master’ scenario.

Two of the hypotheses tested in this thesis (i.e. hypotheses #1 and #2) could relate to the first category of environmental variation mentioned above, in other words relatively rapid and sudden change. Thus, in Chapters 5, 6 and 7, the response of the model species, following the multiple plant introductions that took place in New Zealand, was investigated. The introduction of exotic pastoral plants, particularly white clover, has resulted in the establishment of a differential relationship between Costelytra species and these plants. As discussed in Chapter 7, it appears that C. zealandica was pre-adapted to be unaffected by the potential detrimental effects of clover defences, and, in contrast with its congener, could benefit from them. Although the detail of the mechanism underpinning these results is yet to be investigated, it appears to be based on pre-adaptation rather than on recent acquisition as the result of an “arms race” between the insect and its host (Chapter 7). Such pre-adaptation may indicate a high degree of phenotypic plasticity in the invasive species C. zealandica, as proposed in the three scenarios established by Richards et al. (2006) (i.e. Jack-of-all-trades, Master-of-some and Jack-and-master). Although these three scenarios, which are based on the importance of phenotypic plasticity for maintaining fitness across different environments, were developed with regard to invasive plant species, they could readily be applied to animal species. So far, only one study by Knop and Reusser (2012) has referred in their conclusion to these scenarios in relation to an invasive animal species and it seems that no study has yet extrapolated this principle to invasive insect species. In their study, Knop and Reusser (2012) reported that the invasive slug Arion lusitanicus (Mabille) survived better and produced more eggs than its non-invasive congener Arion fuscus (Müller) under stressful conditions, which comprised high temperatures and low food levels. They concluded that the response of the invasive species fitted the ‘Jack-of-all-trades’ scenario proposed by Richard et al. (2006). The studies in this thesis showed that C. zealandica is able to maintain and even increase fitness in a stressful environment, brought about by triggered defences in its host plant (Chapter 7). In addition, C. zealandica populations occurring in native grasslands, appeared to cope as well as the populations collected from exotic pastures in terms of survival and weight gain, when fed with a ‘new’ host plant (Chapters 5 and 6). These results suggest that C. zealandica invasion success might rely on its ability to rapidly take advantage of newly available
resources. Therefore, the results of this thesis fit the ‘Jack-and-master’ scenario in relation to phenotypic plasticity and invasive species as defined by Richards et al. (2006).

The initiation of the process of invasion in native phytophagous insects, in general, may also rely on high levels of phenotypic plasticity, as suggested by the overall results of this thesis (Figure 9.1). Hence phenotypic plasticity, that allows an insect to tolerate a newly encountered host and achieve similar or significantly better fitness on that host compared with its native host(s), may result in the preferential use of this new host, as observed with the feeding preferences of C. zealandica discussed in Chapter 5. In this feeding preference experiment, it appeared that C. zealandica larvae, which were sampled from exotic pastures, preferred to feed on their exotic host white clover, rather than on their native host. As far back as the 19th century, Walsh (1864) suggested that a host shift resulting from a change in host preference might contribute to new host race formation in phytophagous insect species. The results of the artificial host-shift performed in Chapter 6 support this theory. This study suggested the existence of two distinct biotypes/host-races in C. zealandica resulting from the successful ecological host-shift of one population of this species onto introduced pastoral plants.

In the past, allozyme electrophoreses have been widely used in insect population studies (Richardson et al. 1986, Loxdale and Den Hollander 1989). For example, Gotoh et al. (1993) demonstrated the existence of high genetic differentiation in the PGI enzyme system via allozyme electrophoresis, showing that the two-spotted spider mite, Tetranychus urticae Koch, occurring both on tomato and cucumber, were actually two distinct host races. In a similar way, the analyses of the electrophoresis profiles of the PGI enzyme system, made on various populations of C. zealandica (Chapter 8), could have provided further evidence of the existence of several host-races in this species. Unfortunately, in Chapter 8, both populations of C. zealandica investigated were collected from exotic pastures, thus no comparison between different host-races / biotype, as defined in Chapter 6, could be made. Hence, any PGI-allozyme variation that could have been detected between the two populations studied could not be linked with genetic differentiation due to host-race formation. Additionally, these allozyme electrophoresis profiles could also have been used to investigate Richards et al. (1997) results and Travis Glare’s (personal communication, Bio-Protection Research Centre NZ) supposition regarding some degree of genetic divergence between North and South Island C. zealandica. Although such investigation was not performed in Chapter 8, other divergences, based on thermal tolerance, were nevertheless detected between the populations of both islands (Chapter 8). It appeared that the weight gain of the larvae collected from the South Island was significantly depressed by low temperature (i.e. 10°C), while no such effect was detected in North Island larvae.

The third and last hypothesis tested in this thesis (i.e. hypothesis #3), which investigated thermal tolerance in an invasive and non-invasive species pair, relates to the second and third categories.
of environmental variation mentioned earlier. The different temperature regimes that were investigated in Chapters 5 and 8 could either characterise a gradual and long-term change (e.g. temperature variations associated with global climate change) or a more repetitive and predictable change (e.g. seasonal temperature changes). The results of this thesis showed that C. zealandica was more tolerant than C. brunneum to challenging soil temperatures (Chapter 5 and 8). Larval development in terms of weight gain in the invasive species was indeed, far less impaired by the challenging soil temperatures tested, than that of the non-invasive species. Such tolerance could indicate a higher degree of phenotypic plasticity with regard to soil temperature in the invasive species than in the non-pest species. Recently Ju et al. (2013), who investigated the tolerance of an invasive lace bug to high temperatures, stressed that under extreme temperatures, the fitness response of a species may be related to phenotypic plasticity and that this possibility has already been discussed in similar studies with regard to other insect species. For instance, Hawes and Bale (2007) related multiple levels of response (i.e. from molecular to behavioral) to low temperatures of arthropods species in general, to phenotypic plasticity.

In the present study, Costelytra spp. response to challenging temperatures did not appear to be related to the pgI gene and more precisely with PGI allozyme forms expressed, as initially suspected (Chapter 8). In addition to other parameters discussed in Section 8.4 such as small and unbalanced sample size and low allelic variability in Costelytra species, the difficulty of interpreting the PGI electrophoretic profiles could be another reason why no relationship between this gene and thermal tolerance in the studied species was found. Despite these results, other studies have established a link between thermal tolerance and the pgI gene in various species, including cnidarians (Zamer & Hoffmann 1989), beetles (Dahlhoff & Rank 2000) and moth and butterfly species (Karl et al. 2009, He 2010). Therefore, it is still possible that the differential expression of this gene could be involved in the invasion success of some insects by allowing them to extend their range over wider geographical areas than other species. Several studies have successfully linked various forms of PGI allozymes with the expression of heat shock proteins (Hsps), which play important roles in thermal tolerance by reducing stress-induced protein aggregation (Dahlhoff & Rank 2000, Dahlhoff & Rank 2007, McMillan 2005). Possibly additional investigations on Hsps expression in the model species of this thesis, rather than on the PGI enzyme system itself, could help to clearly establish whether the tolerance to challenging soil temperatures observed in the invasive species C. zealandica actually relates to phenotypic plasticity. Indeed, Via et al. (1995) described two distinct classes of phenotypic plasticity and qualified them as ‘allelic sensitivity’, where either some alleles could be expressed in different environments with varying effect on the phenotype, and ‘gene regulation,’ where regulatory loci may cause other genes to be turned on and off in response to particular environment. Hence, the pgI gene might be involved in the expression of Hsps through gene regulation as described by Via et al. (1995), which in this case would indicate that thermal tolerance is related to phenotypic plasticity.
9.4. Summary and concluding notes

**So why do some species become invasive in their native range?** The present thesis offers an overview of the explanations and the mechanisms responsible for the invasion success of *C. zealandica* in its native range in exotic pastoral systems. The most important conclusion based on the work presented in this thesis is the potential key role played by phenotypic plasticity in *C. zealandica*’s invasion success, which possibly extrapolates to phytophagous insects in general (Section 9.3).

Of the three main working hypotheses that this thesis investigated (Section 1.6.1) all were accepted as more likely to be true with the use of *C. zealandica* and *C. brunneum* as the model species. As summarised in Figure 9.1, a phytophagous insect that has higher fitness on a new host species is better able to overcome the defences of that new host compared with a closely related non-pest species (Hypothesis #2). This was observed in the present study as most likely a pre-adaptation by which the invasive species was able to benefit from the defences of the new host plant (Chapter 7). This pre-adaptation -or similar capability relying on high degree of phenotypic plasticity-, as shown in Figure 9.1 might be an important prerequisite to initiate the process of invasion success in some phytophagous insects in their native range. If such species were given the opportunity (i.e. direct access to a new host plant), the insect could consequently develop preferences and higher fitness response on the new host compared with on its ancestral host, in contrast with a closely related non-pest species (Hypothesis #1). In *C. zealandica*, the sporadic opportunities given by the introduction of exotic pastoral plants in some parts of its native range, appear to have also resulted in genetic differentiation and host-race formation (Chapter 6, Figure 9.1). Because the coincidence of the initial occurrence of a new host plant within the entire native range of a given insect species remains putatively low, it is likely that the development of invasiveness in phytophagous insects in general, could, at times, hide the formation and existence of distinct biotypes/host-races (Figure 9.1). Finally, this study also, to some extent, demonstrated that a phytophagous insect that becomes a successful invader on a new host in its native range is more tolerant of a wider range of temperatures, in contrast with a closely related non-pest species (Hypothesis #3), as shown by higher thermal tolerances in *C. zealandica* than in *C. brunneum* (Chapters 5 and 8, Figure 9.1). However, in neither of these species, intraspecific tolerance to challenging soil temperatures appeared to be related to the PGI enzyme system.

The conclusions of this thesis were drawn based on a single pair of model species. Therefore, some of the invasion key traits mentioned above may actually vary from one invasive species to another. Therefore, I believe that the investigation of the mechanisms and the characterisation of novel traits associated with the development of invasive characteristics and with the invasion
success of phytophagous insects in their native range, and more generally in insect species, promises some fascinating future research.

Figure 9.1. Summary of the establishment or non-establishment of invasiveness in *Costelytra* spp. (in red) and a generalised model of phytophagous insects that become invasive in their native range (in black).
9.5. Future research

As mentioned above, this thesis offers much scope for future research. The following topics are derived from specific questions that arose over the course of this research project, and would certainly be worth addressing in future investigations.

• The effect of jasmonic acid increased defences on the model species using different exotic host-plants

It is recognised that herbivore-host plant associations change over the time and more new associations may differ from older ones based on the physiological and functional interplay occurring between insect and plant defences (Heard & Kitts 2012). The findings in the present thesis support the importance and the complexity of such interactions in the process of invasion by any phytophagous insect that adopts a new host in its native range. However, because the investigation of host-plant defences was solely based on one exotic host-plant, further investigations using different hosts could better support the findings made in Chapter 7. Because, novel insect-host plant relationships are believed to often involve close relatives of the native flora used by the insect (Carroll & Fox 2007), the first recommendation would be to conduct the experiment described in Chapter 7 using a grass (e.g. Lolium perrenne) instead of a legume as host-plant. Furthermore, the conclusions drawn in Chapter 7 suggest the possible implication of some benefits from plant phenolics in the invasion success of C. zealandica. Therefore, the second recommendation for future research would be to quantify phenolic expression in white clover and to investigate the presumed link between phenolics and increased fitness performance in this insect.

• The thermal tolerance of invasive species based using different molecular methods

As discussed in Chapter 8 and in Section 9.3, further investigation regarding the PGI enzyme system and thermal tolerance in invasive species would be worthwhile. Three research directions could be taken by conducting similar investigations as those described in Chapter 8, but alternatively by, (1) directly assessing the genetic variation of the Pgi gene rather than the expression of PGI allozymes, (2) using another support medium as suggested in Section 8.4, or (3) investigating the expression of Hsps rather than that of PGI allozymes (Section 9.3).
Host races are often considered as intermediary stages towards speciation (Downey & Nice 2011). As suggested in Chapter 6, it is possible that the host-race formation detected in *C. zealandica* will, at some point, lead to speciation. This species, which has not yet split into two, could consequently be an interesting model to study the process of speciation, which according to the Marie Curie SPECIATION Network (Butlin et al. 2012) needs to be reinvestigated.

Additionally, the use of several other invasive / non-pest congeneric model species to investigate some of the above points would complement the findings of the present thesis. Such models could be chosen from the scarab family (Table 1.2), although the investigation of other insect orders and Coleoptera families would show a wider applicability of the results obtained in this thesis. Finally, similar experiments to those carried in Chapters 5, 6, 7 and 8 could also be performed using the adult stage of the species model, which may further support the results of this thesis.

To conclude, there is one last question that I would like to see addressed in the future,

*Are these native insect invaders more likely to establish abroad if given a chance to do so, than a non-invasive native species?*
References


Appendix 1

Non-exhaustive list of native invasive insects in New Zealand — based on R.R Scott 1984

<table>
<thead>
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<th>Order</th>
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<th>Number of species</th>
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<td>Blattidae</td>
<td>1</td>
</tr>
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Appendix 2

Short meta-analysis to characterise imbalance between exotic and native insect invaders studies

**IN A NUTSHELL...imbalance between exotic and native insect invaders studies, a meta-analysis**

**Introduction:** Quantifying the imbalance between the study of exotic and native insect invaders can deeply demonstrate the gaps existing in our knowledge and research direction in term of biological invasion.

**Material & methods:** To acquire a quantitave snapshot regarding exotic and native insect invaders studies over the last decade, we partitioned synonymy of lexical terms, that characterise this topic, into the three following categories.

<table>
<thead>
<tr>
<th>Lexical categories</th>
<th>Synonymy</th>
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<tr>
<td>Exotic</td>
<td>exotic, alien, non-native, introduced</td>
</tr>
<tr>
<td>Native</td>
<td>native, endemic, indigenous, local</td>
</tr>
<tr>
<td>Invasive</td>
<td>invas*, pest</td>
</tr>
</tbody>
</table>

A database was created for the last 13 years (2000–2013) by conducted searches in Web of Knowledge in the three most representative journals of insect invasion studies (i.e. Biological Invasions, Biological control and Environmental Entomology) by searching for term associations of the defined lexical categories.

**Results:**

More than 12% of the research articles relative to insect invasion, in the three selected journals, clearly mentioned one (or several) term combination(s) defined in this study to characterise an exotic invasive species, while only less than 0.2% mentioned those defined to characterise a native invader.

**Conclusion:** Focus in insect invasion studies appears to be on average 93 times more on exotic invaders than it is on native invasive species. This net gap attests the existence of mis-investigated concepts in biological invasion research.
Appendix 3

Cellulose acetate electrophoresis optimisation

General advices and example of the PGI enzyme system in *Costelytra* species

• *Task #1 – Determination of the direction of the migration*

The migration direction of the enzyme system needs to be determined to select an appropriate loading zone on the cellulose acetate plate and an adequate position of this plate in the electrophoresis tank (see Hebert et al. 1993 for details). Most enzymes migrate toward the anodal electrode of the electrophoresis tank due to their net charge (Wilkinson 1970, Richardson et al. 1986). The PGI appeared in several studies to migrate in the opposite direction (Wilkinson 1970).

*Our results – the PGI enzyme system migrates toward the cathodal electrode in both Costelytra species studied, consequently extracts were loaded near the centre of the cellulose acetate plate for the rest of the study.*

• *Task #2 – Optimization of the migration and separation of allozymes*

Enzyme movement through cellulose acetate gels depends on (1) the nature of the charged particle itself, (2) the intensity of the electric field and on (3) the pH of the electrode buffer. (Hellena Laboratories 1976).

In general, high pH buffers provide a better separation of the allozymes than lower pH buffers. On the other hand, many enzymes are more stable at lower pH (Richardson et al. 1986).

*Our results – after trying three different electrode buffers, Tris-Glycine buffer at pH 8.5, the same buffer lowered to pH 7.5, and Phosphate buffer at pH 7, the first one was retained.*

• *Task #3 – Optimization of the allozyme definition*

Enzymes degrade quite rapidly if not kept under optimal conditions, which will affect the quality of the gel. The addition of preservatives to the extract may help to prevent enzyme degradation. The two following reducing agents can be added to the extraction buffer to stabilize the enzyme structure, 0.1% 2-mercaptoethanol and 0.1% dithiothreitol (Isosaki et al. 2011).

*Our results – no improvement was observed after the addition of the agents to the Tris-HCl extraction buffer. The extraction buffer was therefore used with no addition of reducing agents.*

Depending on the tissues samples, the quantities of enzymes may be variable. The selection of the body part used for the preparation of the extract may affect the concentration of the selected enzyme and thereafter the quality of the gel resolution. On the other hand, too highly concentrated extract may result in imperfect electrophoresis resolutions ((Richardson et al. 1986, Hebert & Beaton 1993)).
Our results – three body parts of the species studied where tested: the last abdominal segment, the head capsule and a leg. The optimal gel resolution was obtained using the abdomen of the larvae in 100 ml of Tris-HCl to prepare the extract.

• Task #4 – Final checks and other advice (adapted from ‘Troubleshooting Cellulose Acetate Gels’ in Hebert et al. 1993)

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Most frequent reasons</th>
</tr>
</thead>
<tbody>
<tr>
<td>No staining</td>
<td>Wrong side of the gel used</td>
</tr>
<tr>
<td></td>
<td>Bands migrating toward the cathodal electrode that run off the gel</td>
</tr>
<tr>
<td></td>
<td>Missing chemical in the stain</td>
</tr>
<tr>
<td></td>
<td>Use of old and/or degraded chemicals</td>
</tr>
<tr>
<td></td>
<td>Poor sample preservation and enzyme degraded</td>
</tr>
<tr>
<td>No migration of bands</td>
<td>Electrodes not submerged in the buffer</td>
</tr>
<tr>
<td></td>
<td>Power supply turned off or unplugged</td>
</tr>
<tr>
<td></td>
<td>Dry wicks or wicks not properly set up</td>
</tr>
<tr>
<td>Enzyme migrates in reverse</td>
<td>Gel not properly orientated in the tank</td>
</tr>
<tr>
<td></td>
<td>Electrodes from the power supply reversed</td>
</tr>
<tr>
<td></td>
<td>Enzyme that migrates cathodally because of their net positive charge</td>
</tr>
<tr>
<td>Enzyme stains weakly</td>
<td>Use of old and/or degraded chemicals</td>
</tr>
<tr>
<td></td>
<td>Extract too diluted</td>
</tr>
<tr>
<td></td>
<td>Not enough extract applications</td>
</tr>
<tr>
<td></td>
<td>Poor quality extract (enzyme partially degraded)</td>
</tr>
<tr>
<td>Enzyme stains too intensely</td>
<td>Too many extract applications</td>
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<tr>
<td></td>
<td>Over-staining time</td>
</tr>
<tr>
<td>Wide fuzzy bands</td>
<td>Plate not blotted properly before application of the extracts</td>
</tr>
<tr>
<td></td>
<td>Use of a dirty applicator</td>
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<tr>
<td></td>
<td>Extract applied as wide rather than thin lines</td>
</tr>
<tr>
<td></td>
<td>Extract not prepared in cold conditions</td>
</tr>
<tr>
<td>Extra unaccountable bands</td>
<td>Dirty applicator</td>
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<tr>
<td></td>
<td>Stain artifacts such as non-enzymatic bands</td>
</tr>
<tr>
<td>Poor migration and separation</td>
<td>Electrophoresis running time too short</td>
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<tr>
<td></td>
<td>pH buffer too low or inappropriate buffer for the enzyme studied</td>
</tr>
<tr>
<td>of the isoenzymes</td>
<td></td>
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</tbody>
</table>
Appendix 4

Research outputs

• Conference abstracts and oral presentations

**Lefort M-C** (2013) *When natives go wild...host shift and host-race formation in the endemic New Zealand beetle Costelytra zealandica (White).* Miss EL Hellaby Indigenous Grasslands Research Trust - Fourteenth Triennial Seminar. Dunedin, New Zealand.


**Lefort M-C**, Worner SP, Glare T, De Romans S & Armstrong K (2011) *Lessons from the diet of a native pest, the grass grub beetle - host shift or host range expansion?* The 3rd combined Australian and New Zealand Entomological Societies Conference. Lincoln University, New Zealand.


**Lefort M-C** (2011) *When natives go wild...* Thr3sis competition. Lincoln University, New Zealand


**Lefort M-C** (2009) *Research proposal - When natives go wild.* Bio-Protection Research Centre Seminar Series. Lincoln University, New Zealand

• Posters


• Internal reports


• Published papers (copies of publications available in Appendix 5)


Appendix 5

Author contributions to the manuscripts associated with each thesis chapter

• Results of Chapter 3 – published as:


Lefort M.-C – designed and performed the experiments, collected the larvae, designed the primers, acquired and interpreted the data, wrote the manuscript*, prepared figures and table, submit the manuscript and addressed the revisions.

Boyer S. – provided experimental/laboratory advices, provided assistance with manuscript preparation**, prepared supporting informations (Gene Bank accessions) and helped to address the revisions.

Worner S.P. – ensured the funding and provided editorial help***.

Armstrong K. – helped with primer design, ensured the funding and provided assistance with manuscript preparation**.

• Results of Chapter 4 – published as:


Lefort M.-C – collected, ID and prepared the larvae, acquired and described the data, performed the statistical analyses, wrote the manuscript*, prepared figures and table, submit the manuscript and addressed the revisions.

Barratt B.I.P. – acquired the data, provided assistance with manuscript preparation** and helped to address the revisions.

Marris J.W. – contributed regent material and/or analyses tool, provided taxonomical advices, provided assistance with manuscript preparation** and helped to address the revisions.

Boyer S. – provided assistance with manuscript preparation**, prepared the figures and helped to address the revisions.

• Results of Chapter 5 – submitted as:

Lefort M.-C. – designed and performed the experiments, collected the larvae, acquired and interpreted the data, performed the statistical analyses, wrote the manuscript*, prepared the figures, submit the manuscript.

Worner S.P. – ensured the funding and provided assistance with manuscript preparation**

Vereijssen J. – provided experimental/laboratory advices, provided assistance with manuscript preparation**

Glare T.R. – provided assistance with manuscript preparation**.

Boyer S. – provided experimental/laboratory advices/help, provided assistance with manuscript preparation**

• Results of Chapter 6 – submitted as:

  Lefort M.-C., Boyer S., De Romans S., Glare T.R., Armstrong K. and Worner S.P. (currently under revision) Invasion success of a scarab beetle within its native range: host range expansion vs. host-shift. PeerJ

Lefort M.-C. – designed and performed the experiments, collected the larvae, acquired and interpreted the data, performed the statistical analyses, wrote the manuscript*, prepared the figures, submit the manuscript and addressed the revisions.

Boyer S. – provided experimental/laboratory help, prepared the figures (revised version), provided assistance with manuscript preparation** and helped to address the revisions.

De Romans S. – collected the larvae, performed the experiments, provided assistance with manuscript preparation**.

Glare T.R. – provided assistance with manuscript preparation** and helped to address the revisions.

Armstrong K.– ensured the funding and provided editorial help***.

Worner S.P. – ensured the funding and provided editorial help***.

• Results of Chapter 7 – submitted as:

  Lefort M.-C., Worner S.P., Róstas M., Vereijssen J. and Boyer S (currently under revision) Responding positively to plant defences, a candidate key trait for invasion success in the New Zealand grass grub Costelytra zealandica (White) (Scarabaeidae: Melolonthinae). New Zealand Journal of Ecology

Lefort M.-C. – designed and performed the experiments, collected the larvae, acquired and interpreted the data, performed the statistical analyses, wrote the manuscript*, prepared the figures, submit the manuscript and addressed the revisions.

Worner S.P. – ensured the funding, provided assistance with manuscript preparation** and helped to address the revisions.

Róstas M. – provided experimental/laboratory advices, provided assistance with manuscript preparation** and helped to address the revisions.

Vereijssen J. – provided experimental/laboratory advices/help, provided assistance with manuscript preparation** and helped to address the revisions.

Boyer S – provided assistance with manuscript preparation** and helped to address the revisions.
* Wrote the manuscript – preparation of first and subsequent drafts and incorporation of the different co-authors suggestions to the manuscript

** Assistance with manuscript preparation – writing assistance (e.g. suggestions, direct writing input in introduction and/or discussion section(s), provision of new references…), grammatical assistance and stylistic suggestions

*** Editorial help – grammatical assistance and stylistic suggestions