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# Investigation of the relationship between the PGI enzyme system and scarabs fitness response to temperature as a measure of environmental tolerance in invasive species

In the field of invasion ecology, the determination of a species environmental tolerance, is a key parameter in the prediction of its potential distribution, particularly in the context of global warming. In poikilothermic species such as insects, temperature is often considered the most important abiotic factor that affects numerous life-history and fitness traits through its effect on metabolic rate. Therefore the response of an insect to challenging temperatures may provide key information as to its climatic and therefore spatial distribution. Variation in the phosphoglucose-6-isomerase (PGI) metabolic enzyme-system has been proposed in some insects to underlie their relative fitness, and is recognised as a key enzyme in their thermal adaptation. However, in this context it has not been considered as a potential mechanism contributing to a species invasive cability. The present study aimed to compare the thermal tolerance of an invasive scarab, *Costelytra zealandica* (White) with that of the closely related, and in part sympatrically occurring, congeneric non-invasive species *C. brunneum* (Broun), and to consider whether any correlation with particular PGI phenotypes was apparent. Third instar larvae of each species were exposed to one of three different temperatures (10, 15 and 20°C) over six weeks and their fitness (survival and growth rate) measured and PGI phenotyping performed via cellulose acetate electrophoresis. No relationship between PGI phenotypes and fitness was detected, suggesting that the PGI may not be contributing to the invasion success and pest status of *C. zealandica*.



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## Introduction

Understanding environmental tolerance is a key factor in predicting a species potential geographic and ecological range. This in turn is important for the successful management of invasive species. In insect species thermal tolerance is especially important, with extreme temperatures known to affect their development and influence their population dynamics (Wallner 1987, Clarke 2003, Sinclair et al. 2012). Direct effects on metabolic rate impact on a number of life-history and fitness traits (Clarke 2003, Karl et al. 2008), among which survival and growth (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 of three river drainages tested, which was exposed to subzero night-time air temperature. Similarly, Kallioniemi and Hanski (2011) reported low survival rates with larvae of the Glanville fritillary butterfly *Melitaea cinxia* (Linnaeus) subjected to low temperature stress. High temperatures can be equally detrimental as demonstrated by Papanikolaou et al. (2013), where development of the immature stages of the 14-spotted ladybird beetle *Propylea quatuordecimpunctata* (Linnaeus) was greatly impaired and high mortality rates recorded at the two highest temperatures tested in their study. In fact, temperature is considered the most important abiotic factor affecting the success of phytophagous insects (Bale et al. 2002). Not surprisingly it has also been suggested that invasive species may have a broader and greater physiological tolerance of temperatures than native species sharing the same habitat (for a review see Zerebecki & Sorte 2011). Therefore, knowledge of the effect of challenging or extreme temperatures on invasive insect species could provide key information towards developing spatial and climatic distribution projections for a range of risk assessment applications, and particularly in the context of global warming.

An opportunity to test this is presented here by a comparison of the invasive scarab *Costelytra zealandica* (White) (Scarabaeidae: Melolonthinae) with the closely related non-invasive species *C. brunneum*. These insects are endemic to New Zealand and also occur sympatrically in several places (Given 1966, Lefort et al. 2012, 2013). The extended geographical occurrence of *C. zealandica*, and its severe negative impact on agro-ecosystems, suggests that it has reached a high degree of invasiveness within its home range. In fact, based on early observations, this species seems to have become so widespread that it is only absent from a few remote locations of New Zealand (Given 1966) and not reached the status of invader for few others (East et al. 1981). This greatly contrasts with the restricted geographical range of *C. brunneum* which remains confined to a few patchy areas throughout New Zealand, essentially located in the New Zealand Southern Alps (Lefort 2013).

The widespread distribution of *C. zealandica* in New Zealand is likely to be, in part, related to its tolerance of the wide range of soil temperatures within the array of those encountered throughout New Zealand (Lefort 2013), from sun-baked pastural environments to alpine reaches. As recently suggested by Sinclair (2012) for numerous insect species, this might be made possible if *C. zealandica* has a high degree of phenotypic plasticity enabling it to perform under such variable conditions. Certainly the initial spread of *C. zealandica* within its native range would have been a consequence of the widespread cultivation of exotic host plants such as ryegrass and white clover (Lefort et al. 2014). However, it would also have possibly required the species to adjust to an expanded range of soil temperatures in a relatively short time frame. Consistent with this is the conclusion of Stillwell and Fox (2009), that the differential responses of the seed beetle *Stator limbatus* (Horn) to temperature, which impacted on survivorship, body size and fecundity, was due to a high degree of phenotypic plasticity, rather than on genetic adaptation resulting from long-term evolution. That temperature may also have had a direct influence on the differences in the distribution of *C. zealandica* and *C. brunneum* is corroborated by the empirical study of Zerebecki & Sorte (2011) on temperature tolerance and stress proteins, which concluded that invasive species tend to live within broader habitat temperature ranges and higher maximum temperatures.

Several enzyme systems have been successfully linked to, or are suspected to play a key role in, animal physiological tolerance to temperature. Lactate dehydrogenase-B (LDH-B), for example, 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 have been related to warm temperature adaptation in blue mussels (Fields et al. 2006), and similarly the isocitrate dehydrogenase locus Idh-1 exhibits significant correlations between allele frequencies and temperature in several species (for a review see Huestis et al. 2009). In invasive species, Hanski and Saccheri (2006) have suggested that the metabolic enzyme system phosphoglucose-6-isomerase (PGI) could play a key role in the expansion and delineation of 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). Through this unique biochemical situation, covariation patterns between *Pgi* genotypes and individual fitness performance or life-history traits are considered likely to arise (De Block & Stock 2012). In fact, phenotypic variability of the PGI enzyme system has been correlated many times to insect fitness performance, and several such studies on the Glanville fritillary butterfly might also be relevant to success of an invasive species. For example, Haag et al. (2005) established that genetic variation in *Pgi* 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 a significant effect on the growth of local populations. The link between lifespan duration and the PGI phenotype showing high dispersal capacity was also demonstrated (Saastamoinen et al. 2009). However, of relevance 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). As such, it has been characterised as the best-studied metabolic enzyme in a recent review of variation in thermal performance in insect populations (Sinclair 2012). Despite this, this enzyme system has never been analysed in a comparative study involving invasive versus non-invasive insect species.

As part of a wider investigation into invasiveness of phytophagous insects, this study aimed to test the hypothesis that *C. zealandica* is more tolerant of a wider range of temperature than the closely related and co-occuring non-pest species *C. bruneum*, thus facilitating its establishment over a wider geographic area, and to investigate whether particular PGI-genotypes are related to individual fitness advantage when exposed to challenging soil temperatures.

## Material and methods

### *Insect sampling and identification*

Young, actively feeding, third instar larvae were collected; one population of *C. zealandica* from the South Island of New Zealand (Hororata, 43°32'17''S 171°57'16''E) and from the North Island of New Zealand (Te Awamutu, 38°09'95"S 175°35'07"E), and one population of *C. brunneum* from the South Island of New Zealand (Castle Hill, 43°12'20''S 171°42'16''E). The three collection sites were respectively labeled A, B and C. Larvae were identified to species based on the methodology described in Lefort et al. (2012, 2013). Fewer *C. brunneum* were able to be found compared to *C. zealandica*. Prior to experimentation all larvae were tested for amber disease, which commonly occurs in *C. zealandica*, as described in (Jackson et al. 1993) and only healthy larvae were used.

### *Survival and growth response 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 (NZ Meteorological Service 1980). Because of the univoltine nature of the *Costelytra* species life-cycle (Atkinson and Slay 1994), feeding third instar larvae are rarely exposed to soil temperatures below 10°C for long periods. Therefore 10 and 20°C were used as realistically challenging temperatures within the normal soil temperature range for these species, while a 15°C standard laboratory rearing temperature (Lefort 2013) 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 at which each larva was reared individually as described in Lefort et al. (2014). All larvae were fed ad libitum with chopped roots of *Trifolium repens* (white clover).

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 and individually stored at -80°C to minimise protein degradation for the electrophoretic study. At the end of the experiment, all the larvae were snap frozen and similarly stored.

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), followed by Least Significant Difference (LSD) post-hoc analysis after exclusion of larvae that died before the end of the six week period. Statistical tests were conducted with R software (R Development Core Team 2009) and GenStat® (GenStat 14, VSN International Ltd, UK).

### *PGI electrophoretic study*

The last abdominal segment of each larva was cut into small pieces on a square glass plate over ice and then ground using an autoclaved plastic rod in 100 µl of cooled extraction buffer (Tris-HCl, pH 8.0) until completely homogenized.

Expression of the PGI allozymes was subsequently examined by cellulose acetate electrophoresis according to the manufacturers instructions (Helena Laboratories, Beaumont, US) and following optimization of the method of Hebert and Beaton's (1993). The final procedure comprised of 10 µl of each homogenate electrophoresed on cellulose acetate plates (Titan® III 76 mm x 76 mm, Helena Laboratories) in Tris-Glycine electrode buffer pH 8.5 at a constant voltage of 200 V and 2 mA for 15 min. A positive heterozygote

control for each *Costelytra* species was run on each plate. Plates were immediately stained with 4 ml of a freshly prepared PGI stain mix (Hebert and Beaton 1993). Staining time was estimated visually and lasted between 1 and 2.5 minutes. Plates were then soaked for 30 minutes in water, blotted dry and preserved by incubating at 60°C for 15 minutes.

Each plate was subsequently digitised using a UVIDOC HD2 (Uvitec Cambridge, UK) and band scoring performed by optimising the definition and aligning the different allozyme profiles obtained using Adobe photoshop CS5 and OmniGraffle 5 Professional (Figure 1). For each population studied, heterozygote and homozygote forms were scored for each population studied; homozygote alleles were assignment as slow or fast based on their relative mobility from the loading zone (Figure 1c).

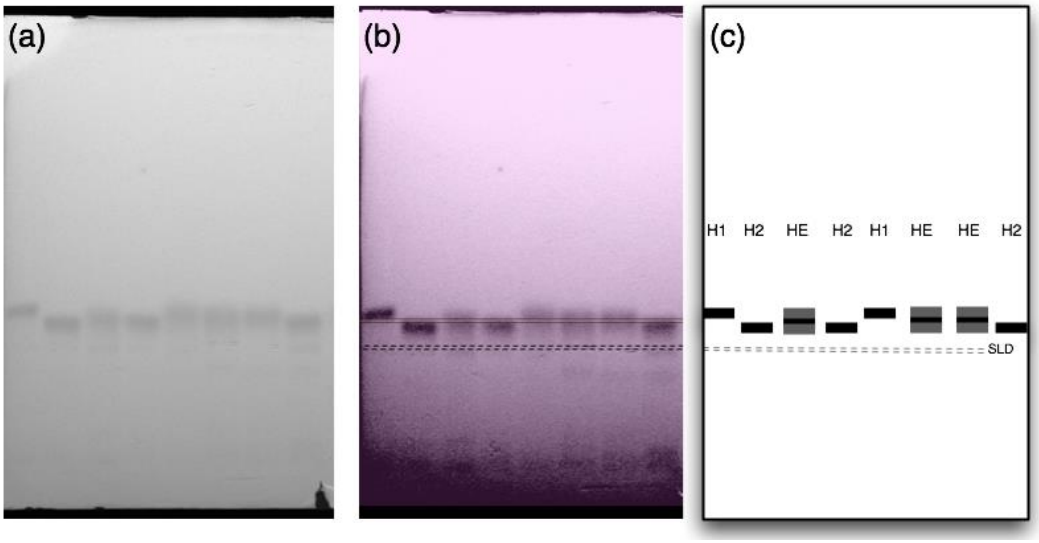


**Figure 1. Example of scoring of a cellulose acetate plate. Where (a) is an original picture of the plate, (b) is a definition optimisation and alignment of the allozyme profiles via Adobe Photoshop CS5 and OmniGraffle 5 Professional and (c) is a representation of the allozyme profiles. H1, fast allele 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) phenotype and temperature regimes, were carried out using Fisher's exact tests (Figure 2).

The PGI phenotype 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 phenotype of each larva used as a co-variate where homozygotes 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. All growth measurements were transformed to percentage weight gain with respect to the initial weight of the larvae prior to the analysis.

# Results

## *PGI electrophoretic study*

The electrophoretic study revealed the existence of only one PGI-locus in both *Costelytra* species (Figure 1). The phenotypes along with their distribution in each temperature treatment are summarized in Table 1.

**Table 1. PGI phenotypes detected by cellulose acetate electrophoresis in *Costelytra zealandica* and *C. brunneum* and their effective distribution in each temperature treatment.**

| | H1 (homozygote - fast allele) | H2 (homozygote - slow allele) | HE (heterozygote) |
|---|---|---|---|
| ***C. zealandica*** (sampling site A) | **n=14** (respectively n= 8, 3 and 3 at 10, 15 and 20°C) | **n=16** (respectively n= 7, 2 and 7 at 10, 15 and 20°C) | **n=36** (respectively n= 9, 14 and 13 at 10, 15 and 20°C) |
| ***C. zealandica*** (sampling site B) | **n=13** (respectively n= 5, 3 and 5 at 10, 15 and 20°C) | **n=43** (respectively n= 17, 15 and 11 at 10, 15 and 20°C) | **n=23** (respectively n= 8, 9 and 6 at 10, 15 and 20°C) |
| ***C. brunneum*** (sampling site C) | **n=10** (respectively n= 1, 5 and 4 at 10, 15 and 20°C) | **n=4** (respectively n= 1, 2 and 1 at 10, 15 and 20°C) | **n=11** (respectively n= 6, 2 and 3 at 10, 15 and 20°C) |

## *Effect of the different temperature regimes on larval growth and survival*

There was no significant differences in larval survival under the different temperature treatments for populations A and C. For population B (*C. zealandica* collected from the North Island of New Zealand), survival was significantly higher at 10°C (100% survival) than it was at 20°C (73% survival) (Fisher's exact test, P=0.0046) (Figure 2).

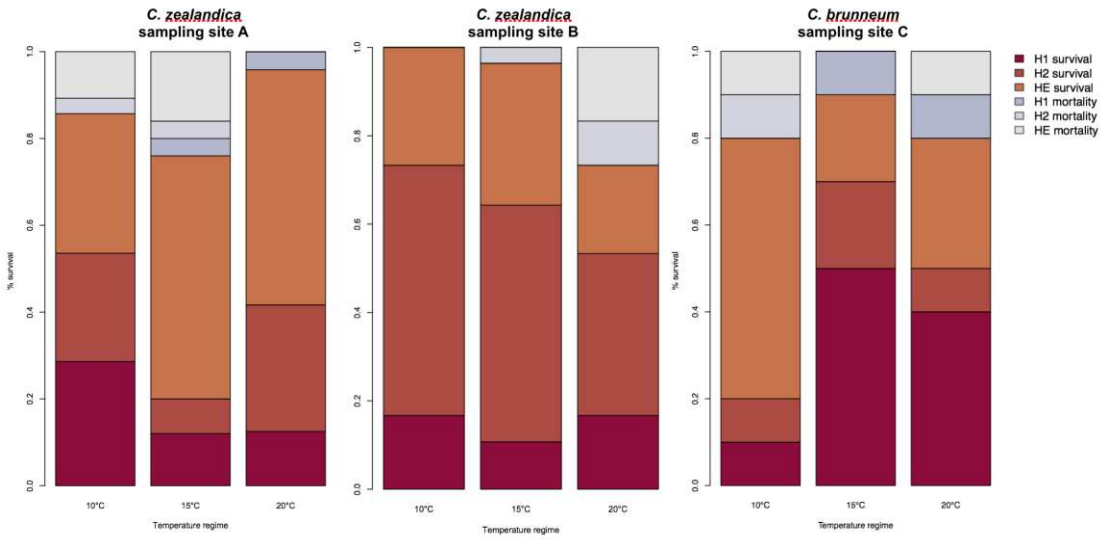


**Figure 2. Percentage of larval survival of *Costelytra zealandica* and *C. brunneum* after 6 weeks of treatment under different temperatures (10, 15 and 20°C) and details of larval survival (dark grey)/mortality rates (light grey) observed for each PGI-genotype detected in each population.**

However, the weight gain of that population was not significantly different at any temperature (Figure 3).

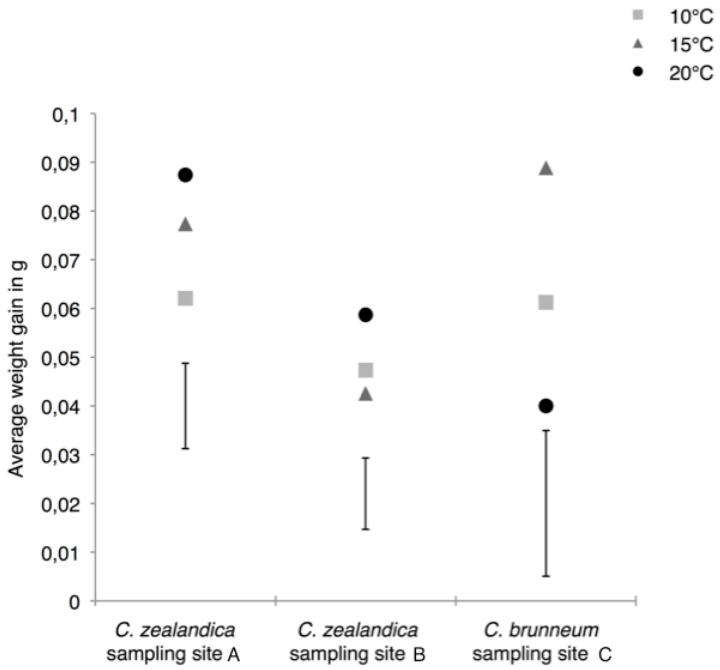


**Figure 3. Average weight gains of the surviving larvae of two populations of *Costelytra zealandica* and one population of *C. brunneum* after 6 weeks of treatment under different temperatures (10, 15 and 20°C). Vertical bars represent 5% Least Significant Difference (LSD).**

In contrast, the weight gain of South Island *C. zealandica* (site A) significantly increased under the highest temperature of 20°C, compared to the lowest temperature (Figure 3), while *C. brunneum* gained, on average, significantly more weight at 15°C compared to the more challenging extremes (Figure 3).

## *Temperature tolerance & PGI-genotypes*

The ANCOVA revealed no significant relationship between the PGI-phenotypes of the larvae with their total weight gain under various temperature regimes (Table 2). Nonetheless, there was a marginally significant effect (at 10% level of significance) of the different temperature regimes on larval growth for the three populations tested (Table 2).

**Table 2. 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.**

| Species (sampling site) | df | *F* | *P values* | Significance |
|---|---|---|---|---|
| ***C. zealandica*** (site A) | | | | |
| Treatment | 2 | 2.42 | 0.098 | marginally ✻ |
| **Co-variate** | 1 | 0.29 | **0.589** | **ns** |
| ***C. zealandica*** (site B) | | | | |
| Treatment | 2 | 2.76 | 0.069 | marginally ✻ |
| **Co-variate** | 1 | 0.05 | **0.830** | **ns** |
| ***C. brunneum*** (site C) | | | | |
| Treatment | 2 | 2.74 | 0.087 | marginally ✻ |
| **Co-variate** | 1 | 0.31 | **0.586** | **ns** |

## Discussion

The main objective of this study 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*'. Towards this the present study aimed at testing whether a relationship between the PGI-genotypes of *Costelytra* species and their fitness response under different temperature regimes exists. However, the results here did not indicate any such correlation, although significant effects of temperature on the larval growth and/or survival of the two species was detected.

Consistent with the latter, Lefort (2013) had previously shown that *C. zealandica* had an improved survival rate at lower rather than at higher temperatures. In the present study this was only apparent for the North Island population, having better survival at 10°C compared with the highest temperature of 20°C. This discrepancy, of no effect here for the South Island population, could be due to the relatively short length of this study compared to the 17 weeks of Lefort, 2013. Additionally, the North Island larvae used here could have been more sensitive to the experimental conditions, because they were subject to a higher degree of disturbance and stress associated with longer transport from the sample site to the laboratory.

In a similar way to larval survival, the temperature effect on larval growth was significantly different between the two populations of *C. zealandica*. 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. 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 have allowed it to successfully establish worldwide under extremely diverse thermal environments (Hoffmann et al. 2003, Morgan & Mackay 2006). There is in fact some evidence of genetic divergence within *C. zealandica* based on the ITS1 rDNA sequences 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, Lefort et al. (2014) have demonstrated that host-race formation in this species might have been instrumental to its invasion success. This may have contributed to the establishment of even further genetic divergence between *C. zealandica* populations throughout New Zealand since that 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.

The non-pest species *C. brunneum*, in contrast to *C. zealandica*, showed significantly impaired, but not lethally detrimental, larval growth under the most challenging temperature regimes, particularly at 20°C. Even though very little is known about the actual distribution of *C. brunneum*, this species seems to prefer mid to high altitudinal ranges (Hoy & Given 1952, Given 1966). In those regions 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 (NZ Meteorological Service 1980)). These observations, corroborate the fact that this non-invasive species is less tolerant than *C. zealandica* to challenging temperatures, particularly higher temperatures, which would be consistent with its failure to extend its geographical range as has its invasive congener.

Because of the potential impact of temperature on the functional properties of metabolic enzymes (Kallioniemi & Hanski 2012), it is often characterised as a key environmental factor affecting the growth and survival of poikilothermic organisms (Kallioniemi & Hanski 2012, Sinclair et al. 2012). Hence, the interest in the expression of various forms of these enzymes, their allozymes or isoenzymes, has considerably increased over recent decades (Karl et al. 2010). Amongst these, 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. Nahrung & Allen 2003, Dahlhoff & Rank 2007). Such polymorphism provides potential for species to vary in their ecological response, including their thermal tolerance (Kallioniemi & Hanski 2011). In the present study, the electrophoretic profiles revealed the expression of two alleles at only one PGI-locus for these *Costelytra* species (Figure 1). However, as the resolution using cellulose acetate technology is not high, there is the possibility that detection of additional alleles was missed because of poor migration and separation of the various allozymes on the gel, or that allozymes of different loci have a similar or highly similar net charge rendering them indistinguishable under these electrophoretic conditions. Alternative higher resolution methods such as mass spectrometry may be needed to confirm this enzyme system is not as polymorphic as it appears in other insect species.

Extensive studies on PGI expression in several butterfly species have demonstrated that enhanced individual performances are correlated with different allelic compositions for various life-history traits. 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 misinterpretation of the electrophoresis profiles, it could explain why no relationship between individual larval fitness responses and PGI genotypes was detected.

Putative heterozygote individuals for this enzyme system might have displayed better weight gain and survival rates under challenging temperatures, if their PGI-associated metabolic pathways provide a greater ability to adapt to a wider range of temperatures than 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 here may be due to the experimental design being driven by the need to perform the temperature experiments prior to sacrificing the larvae for the electrophoretic study. This compromised design, using individual insects of unknown allelic composition, has resulted in small and unbalanced sample sizes as shown in Table 1.

In conclusion, the present study has been unable to support the hypothesis that the *Costelytra* spp. response to challenging temperatures was related to the *pgi* gene and more precisely with PGI allozyme forms expressed by this gene. Small and unbalanced sample size, with respect to allele types, along with the low allelic variability in *Costelytra* species and the resulting difficulties to interpret the electrophoretic profiles could explain why no relationship between this gene and thermal tolerance in the studied species was found. Indeed, 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, we believe that it is still possible that differential expression of this gene could be involved in the invasion success of some insects, 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). Additional investigations on Hsps expression in *Costelytra* spp., rather than on the PGI enzyme system itself, could help to establish whether the tolerance to challenging soil temperatures observed in the invasive species *C. zealandica* somehow relates to the PGI enzyme system. The sympatric nature of the non-invasive and invasive species studied here provided a valuable opportunity to investigate PGI as a marker of invasiveness. There are many other such species pairs such as the

queensland and lesser queensland fruit flies that would serve the same purpose. Therefore we strongly encourage researchers to replicate the experiments described in this paper using such invasive/non-invasive species pairs and to confirm whether or not a relationship exists between the PGI enzyme system and insect fitness response to temperature and with the potential to be used as a measure of environmental tolerance in invasive species.

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