Geographical variation and sexual dimorphism of phenoloxidase levels in Japanese beetles (*Popillia japonica*)

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Phenoloxidase (PO) production can be used as an indicator of pathogen defence in insects. The geographical mosaic of coevolution implies that traits associated with pathogen defence will vary across a geographical range. Bateman's principle implies that the benefit of increased defence levels is greater for females than males. To test both of these hypotheses, we sampled four populations of Japanese beetles, *Popillia japonica*, across Vermont: two were from locations using biological pest control, and two locations did not use biocontrol. We quantified defence levels (PO) and parasite resistance in males and females from each population. Populations from areas with biocontrol exhibited greater PO production, females produced more PO, populations with higher levels of PO suffered less mortality from pathogen exposure, and PO levels positively correlate with increased mortality in the absence of parasitism. Results support the geographical mosaic of coevolution and the implications of Bateman's principle.

Keywords: phenoloxidase; cost of defence; coevolution; geographical mosaic; Bateman's principle; biological control

1. INTRODUCTION

Insects commonly defend themselves by encapsulating organisms or objects that breach the cuticle. The encapsulation process employed by insects involves the formation of melanin around invading objects or parasites. The ability to effectively encapsulate an invader is principally determined by the activation of the enzyme phenoloxidase (PO), which catalyses the first step in melanin synthesis. Empirical evidence has linked levels of PO production with resistance to parasites (see Barnes & Siva-Jothy 2000; Gillespie *et al.* 2000; Cotter & Wilson 2002). Such evidence linking PO levels to parasite defence mechanisms predicts that levels of PO should positively correlate with parasite defence.

The geographical mosaic theory of coevolution states that variation in selection pressures across a geographical range can lead to significant genetic differences between subpopulations of a species (Thompson 1994). Such differences may include pathogen defence levels. The

importance of PO to immunity makes it a likely trait to show variability within a mosaic of parasite selection pressures.

Selection can also vary by sex. Bateman's principle states that males increase fitness by increasing successful mating events, while female fitness is directly related to their lifespan. The time between fertilization and oviposition is not as variable as the time between male mating events, therefore the number of alleles that a female is able to pass onto the next generation is highly dictated by her lifespan. Because the fitness benefit of living longer is greater for females than for males, females should invest more in immunity than males (Rolf 2002). The Bateman principle suggests that PO should be higher in females than in males.

There is evidence that PO shows genetic variation in a variety of insects (see Fellowes *et al.* 1998; Kraaijeveld & Godfray 1997; Ryder & Siva-Jothy 2001; Cotter & Wilson 2002). The abundance of empirical evidence demonstrating genetic variation for PO levels makes a good case for at least a partial genetic basis for variation in PO levels between populations of Japanese beetles.

Japanese beetles (family: Scarabaeidae) are univoltine and spend most of their life underground as larvae. Mating adults emerge in the summer for four to six weeks. *Paenibacillus* (formerly *Bacillus*) *popilliae* and *P. lentimorbus* (Pettersson *et al.* 1999) are used as biocontrol agents. *Metarhizium anisopliae* has also been documented as a successful pathogen of *P. japonica* (Lacey *et al.* 1994), so mortality as a result of exposure to this entomopathogen reflects the ability of the beetles to encapsulate pathogens that breach the cuticle.

This study addresses three questions. The first question is whether variation in PO levels exists between geographically separated populations of the Japanese beetle *P. japonica*. The second question asks if there is variation between males and females in each population. The third question investigates whether differences in PO could be a result of parasite selection by performing a regression of survivorship after exposure to *M. anisopliae* over PO levels. We tested for a cost of PO production as well as its association with overall defence. The interpretations assume PO is correlated with immunity and that PO is a costly defence (Rigby *et al.* 2002). PO is an immune defence for a wide variety of taxa, including arthropods, and also generates toxic intermediates (see Nappi & Ottaviani 2000), thus high levels might be selected against. In addition, pleiotropy might affect PO quantities because PO also plays a role in egg production and synthesis of the insect cuticle. Because melanization is a key process of insect immunity, and PO levels directly correlate with melanization levels, PO should be correlated with resistance against *M. anisopliae*.

2. METHODS

(a) Collection and maintenance of *Popillia japonica*

On 18 July 2001, *P. japonica* were collected from pheromone traps at four locations across Vermont. Locations were chosen because they represented geographically separated populations. The geographical separation, coupled with probable different selection pressures at each locale was hypothesized to represent a geographical selection mosaic. Two populations were sampled from golf courses in Burlington and Stowe where the biocontrol agent *P. popilliae* was applied yearly in mid-spring. We hypothesize the presence of biocontrol will select for beetles that produce more PO. The other two samples were taken from areas where *P. popilliae* was not applied: a residential lawn in Hydeville and a grassy area in North Hero State Park. *Paenibacillus popilliae* use in each location represented a binary

Figure 1. Production of PO by cohort (open bars, use of biocontrol; shaded bars, no biocontrol).

treatment variable. Population stocks were kept in 3 l containers with 3 g of fresh rose leaves, *Rosa rugosa*, that were replaced every 48 h. Males were distinguished from females by the sharp tip on the foreleg tibia, in contrast to the females' long rounded tip (Vittum *et al.* 1999). All beetles were kept at 20 °C and 70% relative humidity in ambient light conditions.

(b) Phenoloxidase assay

PO was measured 1 day after the populations were collected and quantified according to the methods of Barnes & Siva-Jothy (2000) with the following modifications. Whole beetles, rather than just haemolymph, were homogenized in ice-cold sodium cacodylate buffer. Levels of PO were quantified as the increase in absorbance at 490 nm min⁻¹ during the linear phase of the reaction with 5 mM L-DOPA. Results were multiplied by 1000 and log transformed for normality.

(c) Pathogenicity assay

Metarhizium anisopliae was obtained from the USDA-ARS collection, Ithaca, NY, USA. The strain (ARSEF 1080) was isolated from larvae of the corn earworm *Heliothis zea* in Gainesville, FL, USA. Cultures were maintained on Sabouraud dextrose agar supplemented with 10 mM chlortetracycline and grown at 25 °C and 70% relative humidity in the dark (Jones *et al.* 1996). Conidial suspensions were prepared as described in Jones *et al.* (1996) to achieve concentrations of ca. 7.65×10^5 , 3.82×10^4 , 3.82×10^3 and 3.82×10^2 conidia ml⁻¹ of 1% Tween.

Cohorts of 50 males and females were chosen from each population for the pathogenicity assay. From each cohort, 10 individuals were exposed to a solution with no conidia, and each of the four conidial suspensions of varying concentrations were applied to 10 beetles. Beetles were exposed to the solutions by individually submerging for 5 s. After submersion, beetles were maintained in separate 340 ml paper cups with 5 g of sterilized soil and 1 g of leaves that were replaced every 2 days. Survivorship was recorded every 48 h for 14 days. We verified the cause of death by placing the corpses in separate humidity chambers and observing for the characteristic green growth of *M. anisopliae*. Beetles that died from infection by *M. anisopliae* showed green growth.

(d) Statistical analysis

Compound nested ANOVA was used on log transformed PO levels. Model effects were biocontrol use, location nested within biocontrol use and sex nested within location. The effects of PO and conidial dose on mortality were analysed by fitting a Weibull distribution through the data and analysing the resulting parameters. Analysis was performed using JMP v. 4 (Sall & Lehman 2000).

3. RESULTS

PO levels are illustrated in figure 1. ANOVA (table 1) showed that PO quantity is affected by the presence of biocontrol agents. Beetles from areas where biocontrol measures were used showed higher PO production than beetles from areas with no biological control (5.04 ± 0.18

versus 4.53 ± 0.15). Females produced significantly more PO than males except in Hydeville, where there was no significant difference.

Using the fitted Weibull model, likelihood ratio tests showed significant effects of PO, conidial dose and an interaction (table 2).

All beetle corpses from exposure groups exhibited green growth after 4 days in humidity chambers implying death from *M. anisopliae* infection. Unexposed dead beetles showed no green growth.

4. DISCUSSION

The quantity of PO varied significantly across the geographical range (figure 1; table 1). Given the documented role of PO in insect immunity (Nappi & Ottoviani 2000; Cotter & Wilson 2002), these results suggest another empirical confirmation of the geographical mosaic theory of coevolution. For there to be a geographical mosaic of defence levels within a species, the trait that represents the level of defence should bear a cost (Thompson 1994). The presumable cost associated with producing PO is due to resource allocation and the autotoxicity of PO (Rigby *et al.* 2002). If PO was a cost-free defence, then there would be no selection towards lower levels in the absence of pathogens and all cohorts of *P. japonica* would have had similar levels of PO production representing the universal optimum, that is, very high PO production across the board. PO production is positively correlated with increased probability of death in unexposed beetles (figure 2). A trade-off of PO production is consistent with our results because populations with high PO production suffered higher mortality than low PO producing populations in the absence of pathogens, but less mortality is caused by pathogens in high PO populations. To create a causative link between PO levels and mortality, these traits should be examined in a variety of genetic backgrounds and under controlled conditions.

Costly defence mechanisms create a conundrum when attempting to model the adaptation of host resistance. When no pathogens are present, the relationship between defence levels and fitness is negative. The presence of pathogens reverses the association so that defence levels are positively correlated with fitness (figure 2). Here, the time until death empirically illustrates this theory. Actual empirical demonstration of a costly defence mechanism was not shown until quite recently (Kraaijeveld & Godfray 1997). Accumulating such empirical examples will further the advancement of host-parasite coevolutionary theory (Gemmell & Read 1998). Many models of host-parasite coevolution are built on the assumption that the host defence is costly, which selects for decreased quantities of resistance when pathogens are rare or non-existent. Theory predicts that for costly defence mechanisms, fitness is positively associated with defence quantities in the presence of pathogens and negatively associated in the absence of pathogens. Therefore levels of host defence are subject to selection dependent on pathogen abundance. Because pathogens can vary geographically, monitoring costly pathogen defence levels should provide evidence of a geographical mosaic.

According to Bateman's principle, lifespan should have a much larger effect on female fitness than on male fitness (Roff 2002). Our results are consistent with this

Table 1. Summary of nested ANOVA, with unequal subclass size, on the effects of biocontrol use, sex and location on PO production in Japanese beetles ($n = 32$).

source of variation	d.f.	sum of squares	F-ratio	p-value
biocontrol use	1	3.7435	9.5806	0.0049
location (biocontrol use)	2	7.4174	9.4917	0.0009
sex (location, biocontrol use)	4	8.0133	5.1271	0.0039

Table 2. Effects of PO and conidial dose on mortality using likelihood ratio tests from the fitted Weibull model, interaction term = $(\text{PO} - 4.837) \times (\text{dose} - 1.969)$.

source	estimate	d.f.	χ^2	p
PO	-0.2400	1	7.567	0.0059
dose	-0.0633	1	61.37	< 0.001
PO $\times$ dose	0.0225	1	4.182	0.0409

Figure 2. Mortality of Japanese beetles by PO quantity and intensity of exposure. Parametric regression of time of death after exposure to *Metarhizium anisopliae* versus intensity of exposure (log (conidia per millilitre)) and PO quantity. The regression of beetles exposed to higher doses supports the claim that PO is a defence. The regression for unexposed beetles is consistent with a cost of PO defence.

hypothesis. The results suggest that the evolutionary optimum level of PO production is higher in females than in males. The results here suggest that optimal PO levels are different between sex and location, while providing a solid empirical foundation for future investigations about variation in PO levels and immune function. To make the suggestions of this study more compelling, future research should increase the number of sampling locations within each class of biocontrol exposure.

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