Functional Ecology 2005
19, 1032–1039

The malathion-specific resistance gene confers a sperm competition advantage in *Tribolium castaneum*

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Summary

1. Genes for insecticide resistance are usually traded against other fitness traits. However, in *Tribolium castaneum*, the opposite effect occurs under malathion-specific resistance: susceptible individuals show reduced reproductive success compared with resistant lines.

2. To determine the mechanisms within this unusual relationship, we explored male fertilization success after sperm competition between homozygotes of two isogenic lines that were only either resistant or susceptible to malathion. We also measured key male reproductive traits known to influence sperm competition and reproductive success.

3. Malathion-resistant males were superior sperm competitors than susceptible ones, which explains the rapid proliferation of the resistant genotype. This reproductive advantage was associated with increased ejaculate sperm number and sperm length for males carrying the resistance gene. Conversely, susceptible males have absolutely and relatively larger testis size.

4. Resistance to malathion is conferred by an increase in activity of a qualitatively variant detoxifying carboxylesterase enzyme compared to the 'original' enzyme composition in susceptible populations. Carboxylesterases also play important and conserved roles in sperm development, maturation and possibly fertilization in different taxa.

5. Our findings reveal a potential pleiotropic link between a single gene that codes for both insecticide resistance and influences key male reproductive traits through the regulation of carboxylesterase production.

Key-words: Flour beetle, reproductive success, sperm length, sperm number, insecticide

Functional Ecology (2005) **19**, 1032–1039
doi: 10.1111/j.1365-2435.2005.01055.x

Introduction

Insecticide resistance genes often have pleiotropic effects and influence more than one phenotypic character (McKenzie 1996). Usually, experiments measuring reproductive fitness without insecticide challenge show that individuals carrying resistant genes tend to have lower fitness than susceptibles (which were previously the more common and successful genotypes under no selective pressure with insecticide). When pressure from insecticide challenge is relaxed, there is often rapid selection against those alleles conferring and associated with insecticide resistance so that their frequency quickly declines in the population (Crow 1957; Carrière and Roff 1995; McKenzie 1996). Although the majority of studies of insecticide resistance traits show this pattern (see McKenzie 1996 for review; Foster *et al.* 2003; Berticat *et al.* 2004), a few studies have found examples of species where no fitness differences are observed between resistant and susceptible populations (McKenzie 1996 and references therein; Villatte *et al.* 1999; Oppert *et al.* 2000; McCant *et al.* 2005). More recently, we have been exploring a system in which insecticide resistance is actually associated with a significant reproductive fitness advantage, in the Red Flour Beetle *Tribolium castaneum* (Herbst) (Coleptera, Tenebrionidae) (Haubruge and Arnaud 2001; Arnaud *et al.* 2002; Arnaud and Haubruge 2002).

Tribolium castaneum is one of the most common and important stored-product pests throughout the world (Beeman 2003). In this species, malathion resistance is widespread and the resistant phenotype has almost completely replaced the susceptible one since malathion resistance was first recorded (see Subramanyam and Hagstrum 1996 for a review). Moreover, in studies of the effects of malathion-specific resistance gene on the fitness of *T. castaneum* in the absence of

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insecticide it was found that resistant individuals were equally fit or even fitter than susceptibles (Beeman and Nanis 1986; Haubruge and Arnaud 2001). Recent studies show that resistant female *T. castaneum* were more fecund than susceptible females (Arnaud *et al.* 2002) and that resistant males showed generally higher reproductive success than susceptibles (Arnaud and Haubruge 2002). These two reproductive traits combine positively to increase the relative reproductive fitness of malathion-specific resistant populations of the Red Flour Beetle, compared with susceptible ones. This unusual relationship between insecticide resistance and reproductive fitness in *T. castaneum*, however, requires specific investigation to determine whether resistant individuals achieve higher reproductive success under the more realistic reproductive situation that involves sperm competition. *Tribolium castaneum* is a highly promiscuous species with long-term sperm storage (Schlager 1960; Arnaud, Haubruge and Gage 2001d): sperm competition is therefore an implicit element of reproduction and gene flow, which is of prime importance while examining the mechanism underlying the spread of insecticide resistance genes (Haubruge, Arnaud and Mignon 1997; Arnaud, Callaghan and Haubruge 2001a).

It is also relevant to examine the mechanisms by which resistant males achieve a different level of reproductive precedence. Despite the importance of insecticide resistance spread, few studies have examined which male reproductive traits are specifically associated with insecticide resistance genes (when differences in strain fitnesses exist). Work on mosquitoes shows that resistant males are less successful at achieving mating, under competition, than susceptible ones (Gilotra 1965; Rowland 1991; Berticat *et al.* 2002). It is also reported that males resistant to the *Bacillus thuringiensis* toxins mate at a lower frequency and duration in the Colorado Beetle *Leptinotarsa decemlineata* (Say) (Alyokhin and Ferro 1999). Similarly, resistant male Diamondback Moths *Plutella xylostella* (L.) mate at a lower frequency than susceptibles (Groeters *et al.* 1993). In this study, we examine the unusual positive relationship between resistance and reproductive fitness under sperm competition in *T. castaneum*, while measuring the details of male reproductive traits affecting reproductive and fertilization success: insemination success, ejaculate sperm number, sperm length and testis size.

To minimize any confounding effects on male reproductive fitness arising from the wider genetic background beyond resistance genes, we created specific isogenic strains of both malathion-resistant and susceptible populations specifically for the comparisons and experiment, by crossing into a single susceptible strain. The gene for malathion-specific resistance is codominant and autosomal (Haubruge and Arnaud 2001), arising from a spontaneous mutation that codes for a modification in a carboxylesterase enzyme (Haubruge *et al.* 2002). Isogenic strains were selected for comparison by eight repeated back-crossings of resistant with susceptible strains, resulting in a pair of strains that are genetically identical excepted for the small region of the chromosome containing the locus that codes specifically for malathion resistance. Such isogenic strains allow us to draw more specific conclusions about the genetic basis for fitness traits associated with malathion resistance in *T. castaneum*, and we interpret this unusual potential relationship, in the light of carboxylesterase production, in the discussion.

Materials and methods

THE INSECTS AND SELECTION OF ISOGENIC STRAINS

Two flour beetle strains that were resistant and susceptible to malathion were used. The resistant strain (PRm) was originally derived from grain stores in the Philippines. The susceptible strain (Asm) was derived from storage facilities in the Ivory Coast. Beetles were cultured without pesticide exposure in a dark incubator, at 30 ± 3 °C and 60 ± 5% relative humidity with a mixture of whole wheat flour and brewer's yeast (10/1 wt/wt) as rearing medium. Beetles were sexed as pupae and maintained individually (to ensure their virginity) in small vials (5·5 cm^3) with 0·5 g of the medium. Adults were 1-month-old, and therefore reproductively mature, when used in the experiments.

Both populations had been cultured in the lab for many generations before starting the selection in 1997 (Asm was collected in 1989 and PRm in 1976) so that they could be considered as inbred at all loci or at least highly inbred at most loci, which makes the fitness comparison between the selected isogenic resistant strain and the susceptible Asm reliable.

To eliminate genetic background as a variable in comparing male phenotype reproductive success, we introduced the malathion resistance alleles into a uniform, susceptible genetic background. The Asm and PRm strains were used to make the isogenic strain. PRm beetles were crossed and back-crossed to the Asm susceptible strain for eight consecutive generations. For the first cross and the four consecutive back-crosses, we used resistant males and susceptible females to replace all 'field'-derived X chromosome with those of the susceptible strain, namely Asm. Resistant females were crossed to susceptible males during the four last back-crosses. Before each back-cross, resistant progeny from the previous back-cross were selected using a standard discriminating dose bio-assay (Haubruge *et al.* 1997). Following the last back-cross, homozygous resistant beetles were selected using a dose that killed 80% of the adults and eliminate the heterozygous ones. At each new generation, the presence of homozygous susceptible adults was repeatedly detected with the discriminating dose bio-assay. This procedure was applied until no susceptible adults were detected during four consecutive generations. This selection resulted into the production of two genetically uniform

isogenic strains, except for variation in the area containing the single gene for resistance to malathion. Reproductive fitness of the isogenic strains was tested immediately following this selection. The new resistant strain was termed ISO (Arnaud and Haubruge 2002).

FERTILIZATION PRECEDENCE (P2)

Methods for determining fertilization precedence after competitive matings are detailed in Arnaud *et al.* (2001d). Pupae from stock populations were sexed and maintained as the stock culture but individually isolated. Adults were 1-month-old at the start of the experiments. Males were marked for identification on the elytra. Matings were conducted by placing a pair of beetles in a vial with 0·5 g rearing medium for a 48 h mating period during which adults were free to mate. Females were then placed in a new vial with another male for the same mating period. In half of the replicates, females were first mated by a susceptible male (Asm) and then by a resistant male (ISO). In the second half of the replicates, females were first mated by a resistant male and then by a susceptible one. No individual was used more than once in any experiment. The 0·5 g medium was then added to a 55 mm Petri dish with a further 4·5 g rearing medium. Females were then maintained individually in Petri dishes with 5 g of rearing medium and transferred to a fresh dish with new rearing medium every 3 days for 21 days. This transfer minimized any potential larval competition or cannibalism between age classes. We used only susceptible females in this experiment to provide unambiguous markers of paternity: resistant males produce all resistant offspring and susceptibles produce all susceptible offspring (the same crosses with resistant females will generate all resistant progeny, whatever the paternal genotype). After 45 days, adult progeny of every single female was sifted from each 5 g rearing medium and paternity was assigned using a discriminating dose malathion contact bioassay: all susceptibles die while resistants survive (Haubruge, Arnaud and Mignon 1997). Malathion-specific resistance is codominant (Haubruge and Arnaud 2001) and this test therefore enables the identification of the male parent and hence the degree of fertilization precedence of either male competitor.

We ensured that both males had inseminated the female and therefore that sperm competition had occurred by ensuring that progeny production over the 21 days of oviposition arose from both males (i.e. mean sperm precedence values (P2) of either 0·0 or 1·0 were omitted). We determined the paternal genotype (susceptible or resistant) for every offspring produced and calculated average sperm precedence (P2 = proportional fertilization precedence of the second male, or sperm competition success) over each female's entire 21 days oviposition period. Thirty sperm competition and fertilization precedence trials were conducted, with 15 susceptible (Asm) genotype as the second male and 15 resistant (ISO) genotypes second; one of the latter crosses had to be abandoned due to no evidence of sperm transfer by one male. Across these trials we genotyped an average of 279 offspring for each resistant male's P2 (range 145–365, total 3902 progeny) and 231 offspring for each susceptible male's P2 (range 54–359, total 3460 progeny). Accordingly we ran 29 sperm competition trials and screened 7362 progeny.

MALE REPRODUCTIVE TRAITS

Several male traits linked to fertilization and reproductive success were measured for resistant and susceptible genotypes: (i) insemination success, (ii) ejaculate sperm number, (iii) sperm length and (iv) testis size. All males were between 1 and 3 months of age, to ensure reproductive maturity. Before experiments, males were weighed to the nearest 10^{-4} g with a Sartorius 'supermicrobalance' (Sartorius, Belgium).

INSEMINATION SUCCESS

Male Flour Beetles are highly polyandrous and can re-mate in succession within very brief periods (Haubruge *et al.* 1999). However, males frequently do not produce an ejaculate during mating in *T. castaneum* (Arnaud *et al.* 2001c). To measure realized mating success therefore we measured the rates of successful insemination by determining spermatophore presence. A pair of virgin beetles was placed in a 35 mm Petri dish lined with a filter paper. Beetles were observed at room light and temperature. After the first mating, the female was anaesthetized and dissected to check for spermatophore transfer. When no spermatophore was observed, the male was provided with a new virgin female. This operation was repeated until sperm transfer was observed so that we could measure average number of mating attempts of either genotype until successful insemination. The insemination success of each male was defined as 1 divided by the number of mating to obtain successful insemination (so that a higher value = higher average insemination success). Ten males of each population were screened.

EJACULATE SPERM NUMBERS AND SPERM LENGTH

Spermatozoal traits were measured according to methods detailed in Arnaud *et al.* (2001c). After successful sperm transfer, the entire female reproductive tract was isolated and placed on a cavity slide with 100 µl modified Barth saline (Gage and Cook 1994). The reproductive tract was then ruptured with entomological needles to allow the ejaculate sperm mass to disperse. The sperm-buffer solution was then washed off the slide with saline into a tube and diluted with deionized water into a gently mixed homogeneous solution. Four 20 µl subsamples were retrieved using a micropipette and placed as smears on a flat slide and allowed to air

dry. Every sperm in each of the 20 µl dried smears was counted under 200 × magnification using dark-field phase contrast microscopy (Olympus BX-50 Omnilabo, Belgium). The total numbers of sperm per ejaculate were calculated by multiplying the mean sperm count from each male's four smears by the ejaculate's dilution factor.

Sperm on the dried smears lie in a flat two-dimensional plane on the slide. Dark-field phase contrast images of intact sperm were relayed at 400 × magnification via a JVC video camera (Omnilabo, Belgium) to a flat-screen monitor. The entire length of each intact sperm was traced onto an acetate film fixed to the monitor screen. These images were then measured using a digital map-measurer and lengths converted to micrometres. Ten sperm per male were measured (because of significant intermale variance in sperm length, this sample size will represent a male's mean sperm length Morrow and Gage 2001; Arnaud *et al.* 2001c).

TESTIS SIZE

After a successful mating, the male was subsequently isolated in a vial with rearing medium for 1 week under standard rearing conditions, after which the male was anaesthetized and testes dissected out. The paired testes were isolated by severing their connections with the male reproductive tract within the abdominal cavity. Removed testes were placed on a small preweighed piece of aluminium foil for 24 h in an incubator at 35 °C and then dry-mass determined to the nearest 10^{-4} g with a Sartorius supermicro-balance.

STATISTICAL ANALYSIS

Prior to analysis we checked each data set for normality. The P2 data showed significant departure from normality (Kolmogorov–Smirnov, Asm-Iso $z = 1{\cdot}38$, $P = 0{\cdot}04$, Iso-Asm $z = 1{\cdot}08$, $P = 0{\cdot}19$) and we therefore analysed these using more conservative non-parametric methods. All other distributions showed no departures from normality (maximum $z = 1{\cdot}02$, $P = 0{\cdot}25$) and therefore parametric testing using analysis of variance (ANOVA) were applied. For every trait tested, variances were found to be equal between resistant and susceptible male data sets (Levene's test, maximum $W = 3{\cdot}451$, $P = 0{\cdot}080$). Statistical analysis were performed with SPSS version 11 (SPSS 2001) and Minitab version 13·20 (Minitab 2001) for Windows.

Table 1. Average (± standard errors) male reproductive traits for susceptible (Asm) and resistant (ISO) genotypes of isogenic lines of *T. castaneum*. For details of sample sizes see Materials and methods

Genotypes	Insemination success[1]	Ejaculate size[2]*	Sperm length (in µm)*	Testis mass (in mg)*
Susceptible (Asm)	0·46 ± 0·1	127 125 ± 17 722	902·6 ± 6·5	80·8 ± 1·5
Resistant (ISO)	0·39 ± 0·1	198 244 ± 20 680	922·9 ± 5·9	62·3 ± 1·2

[1]Insemination success is defined as 1 divided by the number of matings to obtain successful insemination.
[2]Number of sperm in a single ejaculate.
*Statistically significant differences (see Results for more details).

Results

FERTILIZATION PRECEDENCE (P2)

As expected from previous studies on sperm precedence in *T. castaneum* (e.g. Schlager 1960; Arnaud *et al.* 2001d; Bernasconi and Keller 2001), we found that the second male to mate in a sequence achieved higher sperm precedence. We also found that resistant males achieved significantly greater fertilization success in competition with susceptible males (Mann–Whitney *U*-test, $z = -1{\cdot}964$, $P < 0{\cdot}05$ see Fig. 1). We therefore demonstrate that, over a 21-day oviposition period, resistant genotypes show superior sperm competitiveness than resistant genotypes in *T. castaneum*. The difference is driven by male insecticide resistance genotype and not cross type or offspring production since we randomized mating order (14 replicates of Asm-ISO and 15 of ISO-Asm) and there were no differences in offspring production from either cross order ($z = -1{\cdot}18$, $P = 0{\cdot}24$).

MALE REPRODUCTIVE TRAITS

Values for male traits of the susceptible (Asm) and resistant (ISO) genotypes are presented in Table 1. Numbers of mating attempts prior to successful insemination did not differ between the genotypes ($F_{1,18} = 1{\cdot}11$, $P = 0{\cdot}305$). However, resistant genotypes produced significantly larger ejaculate sperm numbers than susceptibles ($F_{1,18} = 6{\cdot}83$, $P = 0{\cdot}018$). Resistant males also produced significantly longer sperm than susceptible males ($F_{1,18} = 5{\cdot}42$, $P = 0{\cdot}032$). Absolute testis size was also significantly different between the

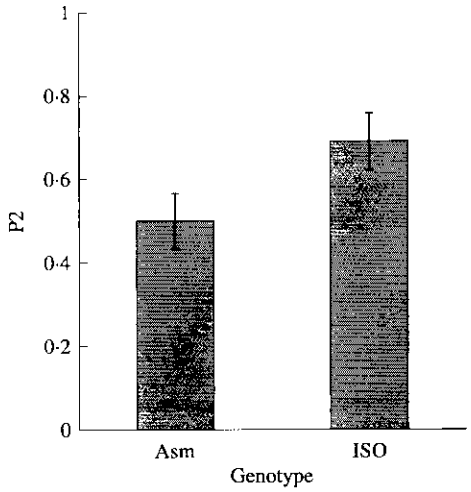

Fig. 1. Mean (± SE) second-male fertilization precedence (P2) in ISO × Asm and Asm × ISO sperm competitions using isogenic lines of *T. castaneum*. $N = 15$ ISO × Asm P2 values (derived from 3460 progeny) and $N = 14$ Asm × ISO P2 values (derived from 3902 progeny).

two genotypes ($F_{1,18} = 94{\cdot}0$, $P < 0{\cdot}0001$), but testis size varied in opposition to ejaculate size and sperm length, such that susceptible males developed larger testes. It is possible that allometry could confound this testis/body weight relationship (Tomkins and Simmons 2002). However, no significant relationship was found between testis mass and body mass in the two populations tested (Pearson's test, $r_s = 0{\cdot}369$, $P = 0{\cdot}295$, $r_s = 0{\cdot}123$, $P = 0{\cdot}734$, respectively, for Asm and ISO). Despite this absence of correlation, we control for body mass by analysing testis mass as a proportion of body mass in an ANCOVA with male size as the covariate, we find that the difference in relative testis mass between the genotypes remains strongly significant ($F_{1,17} = 81{\cdot}5$, $P < 0{\cdot}0001$), with male body mass showing no difference between the two populations ($F_{1,17} = 0{\cdot}62$, $P = 0{\cdot}443$) (the interaction between 'body mass' and 'population' was not significant, $F_{1,16} = 0{\cdot}96$, $P = 0{\cdot}341$).

Discussion

Having produced isogenic lines that differ specifically (and were 'founded' from the same population) in the malathion resistance locus, we show that resistant males are significantly superior sperm competitors than males carrying the susceptible genotype (Fig. 1). Our results are based on detailed screening of 7362 individual progeny from 29 sperm competitions and across a 21-day oviposition period. In addition, all experimental females produced healthy clutches averaging 255 offspring (range 54–365). Our measures of male reproductive traits between the two genotypes show that resistant males also produce significantly more sperm in their ejaculates and these sperm are bigger than susceptible males. It is well established that relative sperm number predicts sperm competition success in insects (e.g. Gage and Morrow 2003) and the correlated variance in ejaculate size between the two genotypes is likely to explain the significant advantage of resistant males in sperm competition. Other studies have shown that increased sperm size may also enhance success in sperm competition (e.g. Miller and Pitnick 2002), but a role of sperm size in sperm competition success requires further work because different studies have shown the opposite effect of a small-sperm-size advantage (Gage and Morrow 2003) and the female reproductive tract may influence the mechanisms of competition (Miller and Pitnick 2002). It is also possible that the difference in sperm competitiveness between resistant and susceptible genotypes and their difference in ejaculate size is not an entirely male-driven phenomenon, but perhaps selected by cryptic female choice (Eberhard 1996). In *T. castaneum*, male tarsal rubbing on the female's dorsum during mating influences that male's subsequent fertilization success (Edvardsson and Arnqvist 2000; Bloch Qazi 2003). Male differential genitalic stimulation ability may also influence their reproductive success (Arnaud, Haubruge and Gage 2001b). Accordingly, there may be male behaviours associated with the resistance locus and/or female active preference for resistant male's sperm (perhaps via cuticular hydrocarbon detection Tregenza and Wedell 1997; Ginzel *et al.* 2003) that explain the significant difference sperm competition ability between the genotypes; variant cuticular hydrocarbon signatures exist between insecticide resistant and susceptible strains (Anyanwu *et al.* 1993, 1997).

Our experiment examined sperm competition success after each male had been given mating access to the female for 48 h, rather than competition between two different ejaculates (this design ensured that true competition between both males' sperm occurred and we only analysed crosses where both males achieved fertilization success.) Accordingly, there may be differences in mating and insemination frequency between two male genotypes that could explain the difference in reproductive success. However, we found no differences in the insemination success of susceptible and resistant males under separate tests, with susceptible males actually scoring a higher success (0·46 mating attempts per insemination) than resistants (0·39). In addition, we also find that there are significant differences in absolute and relative testes masses between the male genotypes and that this varies opposite to ejaculate and sperm size, such that susceptible males (who are weaker sperm competitors) have larger testes. It is generally assumed that testis size relates to sperm production capacity (Tomkins and Simmons 2002; but see Schülke, Kappeler and Zischler 2004). However, the testis also functions in hormone production and some testicular tissue may play a humoral role (Fukai *et al.* 1992; Kirby *et al.* 1997; Tricas, Maruska and Rasmussen 2000; Sharpe 2001) so that the testis size/sperm production capacity relationship may be more complex. In addition, we do not know whether there might be differences in the rate of spermatogenesis between the genotypes, although this appears to be a relatively conserved process, with volume of production dependent on number of active spermatogonia, rather than differences in spermatogenic rate (Clermont 1972). Alternatively, it is possible that resistant genotypes produce a larger ejaculate at less frequent intervals. Longer-term measures of insemination success/frequency (where we detected no difference within this study) may reveal how the relative reproductive capacity of susceptibles and resistants vary overall.

Our findings have important pure and applied implications. *T. castaneum* is a key pest of stored products and malathion resistance has spread notoriously rapidly as an example of proliferation of insecticide resistance (Subramanyam and Hagstrum 1996). Previous studies have shown that malathion-resistant males have higher reproductive success in general competition with susceptible males, perhaps via enhanced mating or fertilization success (Arnaud and Haubruge 2002). It has also been demonstrated that resistant females have generally higher fecundity than susceptibles (Arnaud *et al.* 2002). In this controlled study, we show that

resistant males can achieve higher reproductive success and hence elevated gene flow, because of a superior ability in sperm competition, perhaps as a result of increased ejaculate size. Although sperm competition has been previously investigated as a factor in the spread of insecticide resistance genes (Haubruge *et al.* 1997; Arnaud *et al.* 2001a; Arnaud and Haubruge 2002), this is the first study to experimentally prove the importance of sperm competition in the proliferation of resistance genes. Such a mechanism will have contributed significantly, in this highly promiscuous species, to the rapid spread of the resistant genotype through susceptible populations of *T. castaneum* (Subramanyam and Hagstrum 1996), even in the absence of selective pressure with malathion.

Our findings demonstrate a curious condition, in which extremely important realized reproductive fitness traits are associated with a single locus that codes for a modification in a carboxylesterase enzyme that confers malathion-specific resistance. We produced isogenic lines across eight repeated backcrosses and using the same 'founder' susceptible population for producing the resistant and susceptible genotypes. Furthermore, we only examined males that were homozygous at this locus. How is it possible that such a small genetic variation between isogenic lines for malathion resistance can be genotypically associated with such profound male fitness traits? Either the reproductive fitness traits are controlled by alleles or a gene modifier in close association with the resistance gene, or the different esterase activity in resistant genotypes also influences male reproductive traits. It is possible that any modifier or allelic variability, for reproductive traits, lie in such close association with the resistance gene that backcrossing for isogenic lines conserves these genes alongside the resistance gene. If that is the case, then modifier genes or allelic variants for male reproductive traits may always be conserved through linkage with the resistance locus. However, it seems unlikely that the use of isogenic strains leaves sufficient genetic variation between the two genotypes to explain the differences we see in investment in testes, ejaculates and spermatozoa.

Recent research has found a direct link between carboxylesterase, the protein involved in malathion-resistant *T. castaneum*, and male reproductive function in other invertebrate taxa (Mikhailov and Torrado 2000). The biochemical difference between resistant and susceptible genotypes in *T. castaneum* involves a significant 44-fold increase in the activity of carboxylesterase enzyme which confers resistance to malathion (Haubruge *et al.* 2002). The enzyme is also present in susceptibles, but is qualitatively different, its affinity for malathion is lower and it does not hydrolyse malathion as fast as the enzyme from the resistant insects. Allele differences in *T. castaneum* at a single locus generate this difference in esterase activity, which leads to the significant phenotypic effect of malathion-specific resistance under insecticide stress. In addition to toxin metabolism, the carboxylesterase group is also recognized to play an important role in male reproductive function, specifically differentiation, maturation and emission of spermatozoa (Mikhailov and Torrado 1999, 2000). Research suggests that such carboxylesterases are concentrated as seminal proteins in the male reproductive system of different animal groups (molluscs, *Drosophila* and rodents) and that these proteins confer improved metabolic capabilities and functions on spermatozoa (Mikhailov and Torrado 2000). The recently established biochemical links between carboxylesterase and sperm development and function require much further research to determine precise mechanisms of influence. However, the fact that this enzyme is modified in *T. castaneum* males carrying a single gene difference for malathion resistance (Haubruge *et al.* 2002) provides an obvious, but unusual, physiological mechanism that explains how resistant males achieve superior sperm competitiveness and fertilization success. It is reasonable to question why the expression of such a carboxylesterase has not already evolved in all *T. castaneum* populations (perhaps in tandem with insecticide resistance). Despite the improved reproductive fitness of resistant males and females, we would therefore predict a trade-off at some level within this system from a cost of the modified carboxylesterase production. Perhaps the production of this modified carboxylesterase demands higher metabolic investment to support, so that functions such as mate searching or migration are constrained. Alternatively, in this relatively long-lived insect (129–174 days depending upon strain Soliman and Lints 1975; but sometimes over 2 years, Sokoloff 1974 and references therein), there may be a trade-off with longevity and potentially overall lifetime reproductive success. It is also possible that malathion-resistant insects may be less tolerant or less resistant to pathogen challenge.

Acknowledgements

We are grateful to Chuck Fox and an anonymous reviewer for their helpful comments on the manuscript, to S. Lallemand for providing practical assistance and to Y. Brostaux for his help with statistical analyses.

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Received 10 June 2005; revised 19 August 2005; accepted 3 September 2005