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Constraints on adaptive mutations in the codling moth *Cydia pomonella* (L.): measuring fitness trade-offs and natural selection

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Adaptive changes in populations encountering a new environment are often constrained by deleterious pleiotropic interactions with ancestral physiological functions. Evolutionary responses of populations can thus be limited by natural selection under fluctuating environmental conditions, if the adaptive mutations are associated with pleiotropic fitness costs. In this context, we have followed the evolution of the frequencies of insecticide-resistant mutants of *Cydia pomonella* when reintroduced into an untreated environment. The novel set of selective forces after removal of insecticide pressure led to the decline of the frequencies of resistant phenotypes over time, suggesting that the insecticide-adapted genetic variants were selected against the absence of insecticide (with a selective coefficient estimated at 0.11). The selective coefficients were also estimated for both the major cytochrome P450-dependent monooxygenase (MFO) and the minor glutathione *S*-transferase (GST) systems (0.17 and negligible, respectively), which have been previously shown to be involved in resistance. The involvement of metabolic systems acting both through xenobiotic detoxification and biosynthetic pathways of endogenous compounds may be central to explaining the deleterious physiological consequences resulting from pleiotropy of adaptive changes. The estimation of the magnitude of the fitness cost associated with insecticide resistance in *C. pomonella* suggests that resistance management strategies exclusively based on insecticide alternations would be unlikely to delay such a selection process.
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Introduction

Evolutionary responses of populations to a new environment involving mutations with a large phenotypic effect are likely to entail strong deleterious effects (Fisher, 1958). An adapted form may suffer a disadvantage relative to the ancestral form if, when reintroduced in its ancestral environment, the newly favoured character interferes with its ancestral function: a disadvantage regarded as the cost of pleiotropy (Uyenoyama, 1986). Limits to evolution by natural selection therefore arise if pleiotropic effects associated with novel mutations have negative impacts on other fitness-enhancing components (McNair, 1991). Quantitative genetic models provide critical informations on the genetic relationships that may evolve during adaptation in a new environment, but also translate the impact of the pleiotropic effects of newly selected genes on adapted genetic architectures in terms of fitness trade-offs (Holloway *et al*, 1990; Tienderen, 1997). The adaptation of numerous arthropod populations to environmental toxicity has evolved via the replacement of wild-type alleles with pesticide-resistant alleles through strong directional selection (McKenzie and Batterham, 1994). Pesticide spraying in agro-ecosystems is generally regarded as a major shift in environmental conditions relative to the ancestral pesticide-free conditions. In this context, the theory of Fisher (1958) represents a suitable background for explaining the maintenance of polymorphism at the resistance loci, that is, resistance genes are rarely fixed, in natural pest populations (Coustau *et al*, 2001). Selection against resistance has been supported in field populations by a decrease of resistance frequencies in the absence of pesticide (Curtis *et al*, 1978; Lenormand *et al*, 1999). But because gene flows may also dilute resistance alleles in natural populations, resistance costs have been assessed in the laboratory by comparisons of life-history characters between susceptible and resistant strains (Roush and McKenzie, 1987; Carrière *et al*, 1994). It should be pointed out that this latter method requires that both susceptible and resistant strains share the same genetic background (Amin and White, 1984), and only a few studies have been conducted under such conditions. Another laboratory approach to assessing pleiotropic resistance costs consists in following changes in resistance frequencies in untreated population cages, in which resistant and susceptible alleles are segregating, over several generations (Minkoff and Wilson, 1992; Scott *et al*, 2000). This method favours the homogenization of genetic backgrounds by the creation of linkage equilibrium between coselected deleterious genes and each insecticide-resistant genotype, through recombinations occurring over generations in the laboratory populations.

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In the codling moth *Cydia pomonella*, both enhanced cytochrome P450-dependent monooxygenases (MFOs) and glutathione *S*-transferases (GSTs) have been independently shown to confer resistance to diflubenzuron (Sauphanor *et al*, 1997, 1998), the MFO system being regarded as a major mechanism involved in resistance to this compound relative to GST. Adaptation of this species to environmental toxicity through these two metabolic pathways has recently shown to be associated with pleiotropic costs with respect to reproduction and development (Boivin *et al*, 2001). Here, we investigate the impact of such fitness trade-offs on the evolution of insecticide resistance, through an assessement of the net effect of natural selection in relation to the nature of adaptive changes in *Cydia pomonella* populations. To do this, we follow changes in the frequencies of diflubenzuron-resistant phenotypes in a heterogeneous population under insecticide-free conditions for 10 generations. The magnitude of the fitness trade-offs associated with expression of two mutations involved in the same adaptive response is also explored at the biochemical scale, through the use of enhanced-MFO and -GST activities as phenotypic markers of resistance.

Material and methods

Insects

The field-derived susceptible (S) strain of *C. pomonella* has been mass reared continuously for 20 years in the absence of selection pressure on an artificial diet (Guennelon *et al*, 1981). The resistant strain (Rt) used in this study originated from a field population resistant to diflubenzuron. As a result of the low fertility of wild females, the hybrid Rt strain was produced by crossing males of the resistant population with S females, resistance being already known to be autosomal and codominant in these populations (Sauphanor *et al*, 1998). The hybrid strain was selected by exposure to increasing concentrations of diflubenzuron that induced 50% mortality for the 10 first generations. Exposure of the 20 subsequent generations to a constant diagnostic dose of diflubenzuron (500 ppm) eliminated 100% of the susceptible individuals.

Establishment of a heterogeneous population

Generation 0 (G_0) was initiated by introducing 50 adult males and 50 adult females of each of the S and Rt strains in a population cage, which was maintained under standard laboratory conditions ($25\pm1^{\circ}C$, $45\pm5\%$ RH, 16:8 h (L:D) photoperiod). An average random sample of 1500 neonates in the G_0 progeny was mass reared on an insecticide-free diet to constitute the following generation. The remaining larvae were fed on diet in individual plastic cups ($20\times20\times20\,mm^3$) for phenotypic frequency estimations through both toxicological and enzymatic approaches. This procedure was used over 10 generations. An estimate of the effective breeding size N_e (Wright, 1938) reached in the population before estimating phenotypic frequencies was calculated as follows: $N_e=4(N_mN_f)/(N_m+N_f)$, with N_m and N_f being the numbers of males and females introduced in the population cage, respectively. The mean value of the N_e estimate over the 10 generations was 973. This would prevent the occurrence of any genetic bottlenecks.

Phenotypic characterization with standard toxicological tests

At each generation, neonate larvae were tested with a 250-ppm concentration of diflubenzuron, previously spread on the diet of individual plastic cups. Although this concentration allowed a clear discrimination between susceptible and resistant phenotypes, distinction between heterozygotes and resistant homozygotes was not possible because the heterozygotes survived a 250-ppm concentration applied on the diet. The frequencies of the resistant phenotypes were thus inferred from survival percentages at 250 ppm of diflubenzuron. Tests were conducted under standard laboratory conditions. Mortality was recorded after 7 days of exposure to diflubenzuron and corrected with that of controls treated with distilled water (Abbott, 1925).

Phenotypic characterization with enzyme assays

Enzyme assays were performed on 7-day-old larvae reared in individual plastic cups with diet under standard laboratory conditions without exposure to the insecticide. Cytochrome P450-dependent monooxygenase (MFO) activity. MFO activities were determined by measuring 7-ethoxycoumarin-*O*-deethylation (ECOD) (De Sousa *et al*, 1995). Each larva was cut into two fragments and introduced into individual microplate wells containing 100 µl of 50 mM sodium phosphate buffer (pH 7.2) and 0.4 mM of ethoxycoumarin. The reaction was stopped after 4 h of incubation at 30°C by adding 100 µl of glycine (10^{-4} M, pH 10.4)/ethanol (v/v) buffer. Wells receiving glycine/ethanol buffer before incubation were used as control. Fluorescence was measured using a microplate reader (HTS 7000, Perkin-Elmer) with 380 nm excitation, 465 nm emission filters. Glutathione *S*-transferase (GST) activity. Each larva was homogenized on ice in 70 µl of 50 mM sodium phosphate buffer (pH 7.2) containing 0.4 mM final concentration of phenyl methyl sulphonyl fluoride. The homogenates were centrifuged at 4°C for 15 min at 15 000 *g*, and the supernatants were used as enzyme sources. Protein concentrations of the suspension were estimated by the method of Bradford (1976). The GST activity was determined on UV-microplates with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Each well contained 4 µl of enzyme extract, 184 µl of 50 mM sodium phosphate buffer (pH 7.2), 2 µl of 0.1 M reduced glutathione and 10 µl of 30 mM CDNB. Wells containing identical solutions added to 4 µl of sodium phosphate buffer instead of the enzyme extracts were referenced as blanks. Optical density was recorded at 30°C for 1 min at 340 nm.

Before the beginning of the experiment, measures of both GST and MFO activities were performed in the S, Rt and in the hybrid reciprocal F1 strains (SxRt and RtxS) to evaluate levels of enzyme expression.

Analysis of the stability of resistance expression in the resistant Rt strain, in the absence of insecticide

Simultaneously with the population cage experiment described above, toxicological tests and measures of both GST and MFO activities were performed for seven generations in the absence of diflubenzuron in the Rt

strain. This was done to assess whether fluctuations in biochemical markers in the heterogeneous population could be because of intrinsic fluctuations in enzyme expression in the absence of substrate rather than to a loss of resistance genes.

Data analysis

Variation among genotypes in GST and MFO expression was evaluated with an analysis of variance (ANOVA), as well as the effect of the seven generations on the values measured in the unselected Rt strain (Statview, SAS Institute Inc, USA, 1998). The mean enzymatic activities were compared among genotypes and generations with protected least significant difference (PLSD) Fisher tests. The hypothesis of a fitness cost of resistance assumes a selection coefficient which is applied against resistant phenotypes at each generation. Therefore, one would expect the frequency of the resistant phenotypes (p_R) to decrease logarithmically over t generations as follows:

$$p_{R,t} = p_{R,0}(1-s)^t \quad (1)$$

with $p_{R,0}$ being the initial frequency of the resistant phenotypes, and s the fitness cost coefficient. An estimate of s could thus be inferred from (1) as follows:

$$s = 1 - [(p_{R,t}/p_{R,0})^{1/t}] \quad (2)$$

The assumption underlying (1) was tested using linear regressions performed on the log-transformations of both the toxicological and MFO data. The log-transformation procedure allowed the expression of (1) in the following form:

$$\log(p_{R,t}) = \log(p_{R,0}) + t\log(1-s) \quad (3)$$

with log (1–s) being the slope of regression. Therefore, a covariance analysis (ANCOVA) of the linear regressions performed on the above log-transformations was used to evaluate the significance of any divergence in s estimates between both toxicology and MFO production.

Results

Evolution of resistant frequencies in the heterogeneous population

There was evidence that the proportion of 250-ppm-resistant phenotypes declined in the heterogeneous population. Initial frequencies dropped from 0.77 to 0.24 over 10 generations under insecticide-free conditions (Figure 1).

Enzymatic measures in the S, Rt, and F1 individuals show an overlapping of the range of activities between genotypes (Table 1). Even with lower overlapping for the MFOs, a phenotypic discrimination based on levels of activities could not be achieved. Therefore, MFO frequencies were statistically estimated. The MFO values were normally distributed (K. Pearson test, $P = 0.1$). Thus, if the mean MFO activity (m) of a given phenotype could take the values m_R (mean activity of the resistant phenotype) and m_S (mean activity of the susceptible phenotype) with probabilities p_R and $(1-p_R)$, respectively, the mean MFO activity in the population at generation t could be described as

$$m_t = m_R p_{R,t} + m_S(1-p_{R,t}) \quad (4)$$

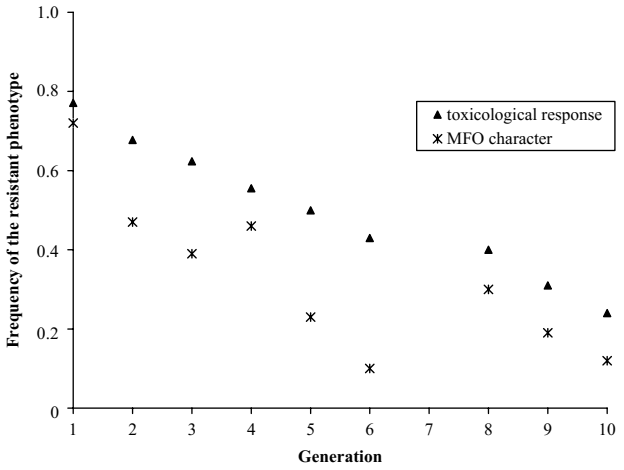

Figure 1 Frequencies of resistant phenotypes in a heterogeneous population of *Cydia pomonella*, held over 10 generations in the absence of insecticide selection. Phenotypic frequencies were inferred from toxicological tests using a diagnostic dose of insecticide, and estimation of metabolic activity of the cytochrome P450-dependent monooxygenase (MFO) character.

The frequency of the resistant phenotypes $p_{R,t}$ was thereby estimated as

$$p_{R,t} = (m_t - m_S)/(m_R - m_S) \quad (5)$$

ANOVA performed on both GST and MFO assays showed that S individuals expressed a mean activity statistically lower than that in the Rt and F1 individuals ($F = 6.032$, df = 2, $P < 0.005$, and $F = 251.254$, df = 2, $P < 0.005$, respectively) (Table 1). The m_R parameter was therefore inferred from the mean MFO activity measured in both the Rt and F1 strains.

Estimated frequency of the MFO character ($p_{R,t}$) dropped from 0.72 to 0.12 over the 10 generations (Figure 1), as a result of a significant decrease in the mean MFO activity in the population (Table 2).

The significance levels of the linear regressions performed on both the log-transformed toxicological and MFO data supported the assumption of (1) ($r = 0.97$, $P < 0.01$, and $r = 0.90$, $P < 0.01$, respectively), that is, the decreasing trend of resistance frequencies for both toxicological responses and MFO production was probably owing to fitness costs (as referred to as s). The linear trend of the responses displayed in Figure 1 may be probably as a consequence of the fact that the time step of the experiment was not sufficiently large to observe an asymptotic behaviour of the frequency estimates, as expected in a logarithmic trend. Nevertheless, according to (2), s values were estimated to be 0.11 and 0.17 for the toxicological responses and MFO production, respectively. According to (3), an ANCOVA analysis performed on the above linear regressions suggested that s was significantly lower for the toxicological responses than MFO production ($F = 100.763$, df = 1, $P < 0.005$).

Although the mean GST activity in the S strain was significantly lower than that in the Rt and F1 strains (Table 1), the overlapping of the ranges of activities between genotypes was too extensive to use the method ($p_{R,t}$) described above. We thus only report the evolution of the mean GST activity in the population (Table 2).

Table 1 Glutathione *S*-transferase (GST) (measured in mg of conjugate formed/mg protein/min) and cytochrome P450-dependent monooxygenase (MFO) (measured in pg of 7OH formed/larva/min) activities in homozygous susceptible (S/S), resistant (Rt/Rt), and reciprocal heterozygotes (S/Rt and Rt/S) of *Cydia pomonella*

Enzyme	*Genotype*[a]	*Mean activity*	*Range of activity*[b]	*Expression ratio*[c]
GST	S/S	1.05a	0.11–2.23	1
	Rt/Rt	1.45b	0.49–2.40	1.38
	S/Rt	1.41b	0.59–2.51	1.34
	Rt/S	1.58b	0.57–2.56	1.5
MFO	S/S	16.27a	1.95–52.03	1
	Rt/Rt	310.84b	121.72–570.16	19.11
	S/Rt	82.98c	24.13–236.87	5.1
	Rt/S	100.54c	28.12–259.07	6.18

For each enzyme, mean activities sharing the same letter do not differ statistically (protected least significant difference Fisher test, $P>0.05$). [a]For each genotype, enzymatic tests were performed on 50 individuals. [b]Lowest–highest values of the measured enzymatic activities. [c]Mean activity of the genotype considered divided by the mean activity of the S/S genotype.

Table 2 Evolution of glutathione *S*-transferase (GST) (measured in mg of conjugate formed/mg protein/min) and cytochrome P450-dependent monooxygenase (MFO) (measured in pg of 7OH formed/larva/min) activites in a heterogeneous population of *Cydia pomonella*, held for 10 generations in the absence of insecticide selection

Generation	*GST*			*MFO*		
	n	*Mean activity*	*95% CI*	*n*	*Mean activity*	*95% CI*
1	128	1.48a	1.33–1.63	149	122.89a	104.51–141.26
2	141	1.44a	1.31–1.57	140	85.92b	71.60–100.23
3	50	1.39a	1.04–1.74	150	74.82b	62.13–87.50
4	127	1.49a	1.35–1.63	133	84.56b	68.40–100.71
5	84	1.59a	1.36–1.82	100	50.19c	40.08–60.30
6	66	1.45a	1.16–1.74	72	36.83c	25.83–47.82
8	95	1.45a	1.26–1.64	97	65.53b	50.84–80.21
9	87	1.49a	1.27–1.71	107	44.85c	40.14–49.56
10	90	1.48a	1.28–1.68	144	34.27c	24.90–43.63

For each enzyme, mean activities sharing the same letter indicate the overlapping of their 95% CI.

Table 3 Evolution of glutathione *S*-transferase (measured in mg of conjugate formed/mg protein/min) and cytochrome P450-dependent monooxygenase (MFO) (measured in pg of 7OH formed/larva/min) activities, and the toxicological response in a homozygous resistant (Rt) strain of *Cydia pomonella*, held for seven generations in the absence of insecticide selection

Generation	*GST*		*MFO*		*Toxicological response*[a]
	n	*Mean activity*	*n*	*Mean activity*	*(n=100)*
1	50	1.45a	50	310.87a	100.00
2	79	1.49a	103	255.18b	100.00
3	75	1.42a	93	271.18b	99.00
4	73	1.47a	101	268.80b	99.31
5	72	1.43a	102	269.47b	99.00
6	77	1.41a	103	270.47b	100.00
7	73	1.42a	101	269.58b	99.31

For each enzyme, values sharing the same letter do not differ statistically (protected least significant difference Fisher test, $P>0.05$). [a]Percentage of survival at 250 ppm of diflubenzuron, corrected by the control mortality.

Enhanced GST activity appeared to remain stable in the population over time (Table 2). However, the mean activity values remained higher than that measured in S/S individuals (see Table 1) ($F=11.827$, df $=1$, $P<0.005$).

Stability of resistance expression in the absence of insecticide substrate

Relaxation of insecticide selection pressure over seven generations did not result in a decrease of resistance in the Rt strain, for both toxicological and enzymatic responses (Table 3). The slight variation in the mean GST activity among generations was not significant ($F=0.228$, df $=6$, $P=0.9675$). There was a significant decrease of the mean MFO activity from G_1 to G_2 ($F=12.675$, df $=1$, $P<0.005$), followed by stability of MFO production from G_2 to G_7 ($F=0.261$, df $=5$, $P=0.9341$). The stability of the toxicological data suggests that such a decrease did not result in a change in the phenotypic response to diflubenzuron on the one

hand (Table 3), and ensured homozygosity at the resistance loci in the Rt strain, on the other hand.

Discussion

Selection against resistance in the absence of insecticide

Our results indicate that the frequency of diflubenzuron-resistant phenotypes decreased over 10 generations in the absence of insecticide. The simultaneous use of biochemical markers of resistance indicated a discrepancy in the evolution of both cytochrome P450-dependent monooxygenases (MFO) and glutathione *S*-transferases (GST) production; the former decreased while the latter remained stable over time under the same insecticide-free conditions. In insects, there is evidence that systems of detoxification such as MFO, GST, and carboxylesterases are inducible by the presence of various insecticides (Terriere, 1983; Feyereisen, 1999). To date, the role of induction mechanisms on enzyme expression still remains a black box in *C. pomonella*. Nevertheless, the relative stability of resistance expression over seven generations in the Rt strain without exposure of diflubenzuron, indicated that the absence of substrate did not alter the expression of modified enzymes in resistant phenotypes. Thus, the loss of the mean MFO activity in the heterogeneous population was a result of a reduction in frequency of the resistance alleles, rather than to a loss of enzyme expression.

Wright (1948) showed that random genetic drift and qualitative fluctuations of selection pressures influence the frequencies of alleles in populations. The decrease in the frequencies of resistant phenotypes in the absence of diflubenzuron may have been a result either of random genetic drift or selection against resistant phenotypes. The latter appear to be the better candidate to explain such a decrease of resistance frequencies. First, random genetic drift introduces stochasticity into changes in gene frequency (Wright, 1948; Uyenoyama, 1986), manifested as random oscillations of the frequency values over time. The statistical analysis of the evolution of the frequencies of diflubenzuron-resistant phenotypes and enhanced-MFO indicated a progressive decrease of resistance frequencies. Second, pleiotropic costs associated with metabolic resistance to diflubenzuron were previously measured in the same strain of *C. pomonella* (Boivin *et al*, 2001), suggesting a selective disadvantage of the resistant phenotypes relative to the susceptible ones. Although we hypothesize that the decrease in resistance frequencies was the expression of a cost, we should point out that, because of the lack of replicate of the experiment, drift effects on resistance frequencies could not be formally precluded in the present work.

To date, manifestations of pleiotropic resistance costs have been well documented with respect to reproduction (Scott *et al*, 1997; Campanhola *et al*, 1991), development (Clarke and McKenzie, 1987) or behaviour (Rowland, 1991; Foster *et al*, 1999). Acquisition of streptomycin resistance in *Escherichia coli* was also shown to affect population growth, by reducing peptide chain elongation rates (Bilgin *et al*, 1992). Nevertheless, despite the abundance of fitness costs in the literature, it is critical to note that negative effects of resistance genes may not be necessarily large, and are not universal (Roush and McKenzie, 1987; McKenzie, 1993; Guillemaud *et al*, 1998). However, the results of this study, in agreement with other work (Chevillon *et al*, 1997; Guillemaud *et al*, 1998), suggests that different selective constraints may be associated with the two enzymatic systems involved in resistance in *C. pomonella*. The fitness cost associated with GST expression appeared to be negligible, because the mean activity measured in the heterogeneous population remained relatively stable over the 10 generations, yet significantly higher than that of the homozygous susceptible individuals. Conversely, the decrease of the frequency of enhanced-MFO phenotypes indicated a costly resistance mutation. Because the toxicological response paralleled that of the MFO character rather than that of GSTs, MFOs appeared to be the major mechanism involved in resistance to diflubenzuron. The GST system may account for resistance as a minor pathway, possibly through epistatic interactions with MFOs that contribute to the enhanced metabolization of the chemical (see Raymond *et al*, 1989). However, GST-based resistance to organophosphates has been shown to involve high fitness costs in the housefly (Roush and Plapp, 1982), which suggests that the magnitude of the fitness costs associated with the acquisition of metabolic resistance might depend on the ratio of expression (relative to the susceptible reference) of the selected pathway.

In all fields of resistance, the expression of fitness costs are generally regarded as the consequence of biochemical and physiological perturbations (Roush and McKenzie, 1987; Bilgin *et al*, 1992; Purrington and Bergelson, 1996). Resistance mechanisms are thought to redirect the enzyme activity that is normally involved in metabolism towards detoxification. Cytochrome P450-dependent MFOs are involved in the metabolization of xenobiotics, but also play a role in the biosynthetic pathways of juvenile hormones and ecdysteroids, endogenous compounds regulating insect growth, development, and reproduction (Feyereisen, 1999). The phenotypic modifications involved in responses to environmental toxicity by generating high metabolization properties may thus interfere with their original function, constituting a physiological burden for mutants in the ancestral untreated environment. Metabolic resistances are also considered to be costly mechanisms, specifically with regard to the regulation mode of their expression (Uyenoyama, 1986; Soderlund, 1997).

Pleiotropic costs and resistance management

The cost of resistance is central to the study of the spread of resistance genes because of its impact on the relative fitness of carriers of such genes (Crow, 1957; May and Dobson, 1986). In this context, deleterious pleiotropic effects of resistance genes emphasize the benefits of the preventive use of nonchemical control methods, which may help to maintain low frequencies of resistance genes in the field. Reversion to susceptibility may be closely linked to the strength of resistance costs and to the rate of immigration of susceptible individuals into the treated area (Tabashnik and Croft, 1982; Carrière and Tabashnik, 2001). Lenormand and Raymond (1998) described a management model leading to the confinement of the resistance genes, by the use of fluctuating intensities of both gene flow and selection (for and against resistance). In south-eastern France apple orchards, the mean frequency of resistance was close to 75% according to

recent field collections (Sauphanor *et al*, 2000). Given that two generations per year can successfully reproduce in this region, the present study suggested that more than 5 years of relaxation of diflubenzuron spraying would be required in order to result in an agronomically effective reversion of resistance in *C. pomonella* populations. We thus postulate that resistance management strategies exclusively based on insecticide alternations would not consistently delay the selection of resistance in this species, suggesting a need for alternative methods to chemical treatments. Although population cage experiments in the laboratory may confer the benefits of an inclusive approach of fitness (Minkoff and Wilson, 1992), the sustainable favourable development conditions allowed by such laboratory conditions may mitigate predictions based on extrapolation to field conditions. Metabolic resistances are known to induce severe deleterious effects in adverse conditions (Chevillon *et al*, 1997; Lenormand *et al*, 1999). Further work is therefore needed to ascertain the magnitude of such a selective disadvantage of resistance under natural conditions, which involve critical events of insect life cycles.

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