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# Within-species variability of the response to 20-hydroxyecdysone in peach–potato aphid (*Myzus persicae* Sulzer)

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**Abstract**

Phytoecdysteroids have been proposed as new tools for controlling crop pests because of their endocrine disruption and deterrent effects on insects and nematodes. There is increasing evidence of variability between taxa in sensitivity to phytoecdysteroids, but the genetic variability of this sensitivity within species is unknown. However, knowledge about this intraspecies variability is required for predicting evolution of the pest's response to new control methods. We assessed the variability of the response of the aphid *Myzus persicae* Sulzer, a major agricultural pest, to 20-hydroxyecdysone (20E). We determined the number of nymphs produced by six clones of *M. persicae* exposed to various concentrations of 20E and the capacity of these clones to detect 20E in choice experiments. High concentrations of 20E significantly decreased the number of nymphs produced for two clones and both increases and decreases in the number of offspring were detected at low concentrations. Two clones significantly avoided food with 20E, while one significantly preferred it, suggesting that 20E does not always act as a deterrent in this species. We conclude that genetic variability in the response to 20E exists in natural populations of *M. persicae*. The consequences of this finding on the sustainability of control methods using 20E are discussed.


*Keywords:* Hydroxyecdysone; Peach potato aphid; Genetic variability; Resistance; Pest species management

## 1. Introduction

Phytoecdysteroids are steroidal compounds widely produced by plant species. They have a similar structure to the ecdysteroids involved in insect development (Lafont, 1997) and it has been suggested that they form part of the plant's defenses against phytophagous animals (Adler and Grebenok, 1999; Lafont, 1997). These molecules are innocuous to vertebrates (Sláma and Lafont, 1995), and are therefore thought to be of potential value for crop pest management (Soriano et al., 2004).

In the last 10 years, empirical studies have been carried out to investigate the effects of phytoecdysteroids on phytophagous species. Many of these studies have focused on the main phytoecdysteroid produced by plants (including cultivated species): 20-hydroxyecdysone (20E). 20E kills and repels several insect (Arnault and Sláma, 1986; Blackford and Dinan, 1997c; Chi DeFu et al., 2002; Kubo et al., 1983; Robbins et al., 1970; Singh and Russell, 1980; Zolotar et al., 2001) and nematode (Soriano et al., 2004) species. Several insect species possess deterrent taste receptors that can detect 20E, allowing them to avoid ingesting this molecule (Descoins and Marion-Poll, 1999; Ma, 1969; Marion-Poll and Descoins, 2002; Tanaka et al., 1994). However, a number of insects have been shown to

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be resistant to 20E (Blackford and Dinan, 1997a–c; Blackford et al., 1996; Tanaka and Naya, 1995). Blackford and Dinan (1997c) suggested that polyphagous insects are resistant to 20E, while oligophagous insects are more sensitive. This would suggest that the use of phytoecdysteroids in agronomic systems may be restricted to a particular set of sensitive species.

Despite the growing body of empirical data, an important aspect of pest responses to phytoecdysteroids has, to our knowledge, never been investigated: no information is available about the genetic variability of the response to phytoecdysteroids in populations or genotypes of a single species. As different plant species produce different amounts of 20E, phytophagous insects are likely to display such within-species genetic variability, as a result of divergent old selection pressures in populations. Measurements of this variability would make it easier to predict the evolution of responses to phytoecdysteroids in pest populations and to estimate the efficiency and sustainability of potential control methods. The use of these molecules may lead to the rapid selection of resistant genotypes if different genotypes in natural populations display different levels of response.

The peach–potato aphid, *Myzus persicae*, (Sulzer) is a major pest that infests a wide range of crop plants. The intensive use of chemical insecticides against this aphid since the 1950s has led to the development of various mechanisms of resistance to the main classes of pesticides (reviewed in Devonshire et al., 1998). This situation is problematic, as fewer and fewer of the available molecules remain efficient against *M. persicae*. An alternative or complementary strategy could be developed, based on the use of natural xenobiotics such as phytoecdysteroids. Preliminary experiments (Alla, unpublished data) have shown that 20E reduces aphid fecundity—a major phenotypic trait determining fitness. As *M. persicae* can develop on several host plants producing various quantities of 20E (for review, see Zeleny et al., 1997), genetic variability in the response to 20E is likely to be found in this species. *M. persicae* is a particularly appropriate model for investigations of this type, because it displays cyclic parthenogenetic reproduction, making it possible to measure phenotypic traits within and between genetically differentiated clones.

In this study, we investigated patterns of genetic variability in the response of *M. persicae* to 20E incorporated into an artificial diet. Six clones were sampled on their primary host. We checked that these clones were genetically different by genotyping them with highly polymorphic molecular markers. We determined the fecundity and survival time of adults exposed to three concentrations of 20E. We also investigated the choices of clones offered food with and without 20E. Based on the results of these two experiments, we analyzed the effects of phytoecdysteroids on *M. persicae* phenotypic traits and the potential risk of selecting insects resistant to this control method.

## 2. Materials and methods

### 2.1. Sampling, identification and maintenance of clones

Within the annual life cycle, cyclic parthenogenetic populations of *M. persicae* reproduce asexually for several generations on peach trees (*Prunus persica*) and on various herbaceous plants, and then produce once sexually on peach trees. Thus, after about 15 generations of clonal reproduction and changes on host plants, adults of the various clones mate on their winter host (Blackman, 1974). Adults of *M. persicae* were collected from peach trees in southern France in early spring 2002, following sexual reproduction. The sampling of individuals on their primary hosts generally ensures that different genotypes are obtained (Guillemaud et al., 2003; Wilson et al., 2002). Clonal lines were constituted from these individuals and maintained in the laboratory on a secondary host: sweet pepper (*Capsicum annuum* L.). We used this host because clones develop well on it in the laboratory (Lapchin et al., unpublished data). Each clone was genotyped, using nine highly variable microsatellite loci—M55, M37, Myz2, M40, S17b, Myz9, M35, Myz25, S16b—described elsewhere (Wilson et al., 2004), to check for the absence of redundant genotypes among the samples. The six clones used in this experiment were chosen at random from the various multilocus genotypes identified.

During the experiments, aphids were fed an artificial diet composed of a mixture of water, sucrose, vitamins, amino acids and trace metal (exact composition available on request). We dispensed 150 μL of this solution between two layers of parafilm. The resulting parafilm sandwich was stuck on the top of a small, cylindrical plastic box (33 mm diam. × 27 mm high). This made it possible for the aphids to move about on the first parafilm layer and to feed on the solution by inserting their stylets through this membrane. Individuals of each clone were transferred from sweet pepper to nutrient solution 2 weeks (about two generations) before the experiment, to reduce maternal effects due to resource modifications.

### 2.2. No-choice experiment

Individual adult females were placed in feeding tubes consisting of a 2 mL microcentrifugation tube, the bottom of which had been cut off and replaced by two layers of parafilm enclosing 15 μL of artificial diet. The same basic diet was used as a control and for 20E dilutions. Two concentrations of 20E (Scitech, 98%) were tested: $10^{-6}$ and $10^{-3}$ mol $L^{-1}$. Although concentrations of 20E in the phloem sap of *M. persicae* host plants is unknown, these values roughly correspond to concentrations of first and highest gustatory response in several Lepidoptera species (Descoins and Marion-Poll, 1999; Marion-Poll and Descoins, 2002). It is also known that concentrations in spinach leaves, one of *M. persicae* hosts, can be very high (Schmelz et al., 1999). We used 15 females per clone and per concentration.

Feeding tubes were placed in controlled environment chambers at 24 °C with a 16:8 h light/dark cycle. Although conditions within these chambers are theoretically homogeneous, we randomized the position of the cages every day to avoid confounding factors that might lead to the incorrect identification of differences between clones. The feeding tubes were changed every 3 days to maintain the nutritive quality of the solution and the experiment was stopped after 9 days.

Each tube was checked daily and the death of females and the number of newly emerged nymphs were recorded. Nymphs were systematically removed from the tube.

### 2.3. Choice experiment

For choice experiments, we used plastic cylinders (33 mm diam. × 27 mm high) covered by two sheets of parafilm enclosing two drops of solution. Each drop (100 μL) was deposited in the centre of a space defined by a narrow strip of double-sided sticky tape placed diagonally across the lower sheet of parafilm. The second sheet of parafilm was placed on top of the first, given a parafilm sandwich in which the two droplets of solution were separated. We introduced a group of 15 aphids into each feeding cylinder. These aphids had free access to both solutions, one of which contained 20E. We tested the choices made by each clone, using two concentrations of 20E: $10^{-6}$ and $10^{-3}$ mol $L^{-1}$. The cylinders were placed and randomly oriented in a chamber with controlled temperature (24 °C) and homogeneous light. The number of aphids feeding on each droplet was counted 24 h after the introduction of the aphids into the cylinder. The experiment was replicated ten times.

## 3. Data analysis

### 3.1. No-choice experiment

We used the total number of nymphs produced by a given female as an estimate of individual performance. This estimate represents the net result of both survival time and fecundity.

A Genmod procedure with a Poisson distribution of the residuals and type III analysis was carried out with Statistical Analysis Software (SAS, 1989). The dependent variable was the number of nymphs produced by a female. The explanatory variables were the main effects "clone" and "20E concentration" and the "clone × 20E concentration" interaction. A significant "clone × 20E concentration" interaction would indicate some clonal (and genetic) variability of the response of *M. persicae* to 20E.

For each clone, we assessed differences between least-square means (option "LSMEANS /PDIFF" in the Genmod procedure) for the number of nymphs produced by females at each 20E concentration. We did not apply Bonferroni correction to the $P$ values obtained, because each planned comparison (Sokal and Rohlf, 1995) related to a specific hypothesis (the response of a particular clone to a particular 20E concentration).

### 3.2. Choice experiment

A Genmod procedure with a binomial distribution of residuals (logit link function) was used to assess "clone" and "20E concentration" effects and their interaction, using the dependent variable "proportion of individuals feeding on the solution without 20E (control zone)". We then tested for deviation from a 1:1 ratio (the null hypothesis corresponding to a random choice) by means of $\chi^2$ tests, using the total number of individuals of each clone for each concentration tested.

We tested whether the preference for a given concentration of 20E was correlated with the number of nymphs produced at this concentration in the no-choice experiment. We then calculated a Pearson correlation coefficient for the correlation between the proportion of individuals of a clone choosing the solution without 20E (rather the solutions containing $10^{-3}$ and $10^{-6}$ mol $L^{-1}$ 20E) and the difference between the numbers of nymphs produced by this clone on a solution without 20E and on a solution containing 20E, in the no-choice experiment.

## 4. Results

### 4.1. Determination of clone genotypes

Fifteen clones of *M. persicae* were genotyped at eight microsatellite loci. The genetic markers used proved to be polymorphic, with two to five alleles per locus. The overall level of genotypic diversity was high, with three (locus S16b) to eight (locus Myz9) genotypes per locus (not shown) and 15 multilocus genotypes among the fifteen clones. Six clones randomly selected from the 15 multilocus genotypes were used for subsequent experiments (Table 1).

### 4.2. No-choice experiment

"Clone" (5 d.f., $\chi^2 = 205.28$), "20E concentration" (2 d.f., $\chi^2 = 62.78$) and "clone × 20E concentration" (10 d.f., $\chi^2 = 86.40$) effects were highly significant ($P < 0.001$ in all cases), indicating clear clonal variability in the response to 20E.

Overall least-square means of the number of nymphs produced were significantly higher for $10^{-6}$ mol $L^{-1}$ 20E than for 0 and $10^{-3}$ mol $L^{-1}$ 20E ($P < 0.001$ in both cases) and were significantly lower for $10^{-3}$ mol $L^{-1}$ 20E than in the absence of 20E ($P = 0.015$).

Differences between the clones are illustrated in Fig. 1. The global trend described above was observed in clones 5 and 6, which performed better at a 20E concentration of $10^{-6}$ mol $L^{-1}$ than at 0 mol $L^{-1}$ ($P = 0.032$ and 0.012, respectively) or $10^{-3}$ mol $L^{-1}$ 20E ($P < 0.001$ for both clones), and better in the absence of 20E than in the presence of $10^{-3}$ mol $L^{-1}$ 20E ($P < 0.001$ for both). Clones

Table 1
Microsatellite genotypes of the six clones of *Myzus persicae* used in the study

| Clone | M55 | M37 | Myz2 | M40 | S17b | Myz9 | M35 | Myz25 | S16b |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 119 127 | 157 163 | 179 203 | 125 232 | 134 168 | 196 222 | 186 186 | 119 144 | 195 197 |
| 2 | 119 119 | 157 163 | 179 179 | 123 129 | 168 170 | 210 222 | 186 186 | 123 123 | 195 197 |
| 3 | 127 129 | 155 157 | 179 191 | 123 135 | 168 168 | 208 210 | 182 186 | 119 144 | 195 197 |
| 4 | 119 121 | 163 165 | 179 203 | 125 125 | 164 168 | 196 222 | 186 186 | 125 144 | 195 197 |
| 5 | — | 157 165 | 179 203 | 125 125 | 164 168 | 196 222 | 186 186 | 123 144 | 195 197 |
| 6 | 127 127 | 157 163 | 203 203 | 129 135 | 168 168 | 196 196 | 186 186 | 119 144 | 195 197 |

For each microsatellite locus, the size of the two alleles (in base pairs) is given. —: missing values.

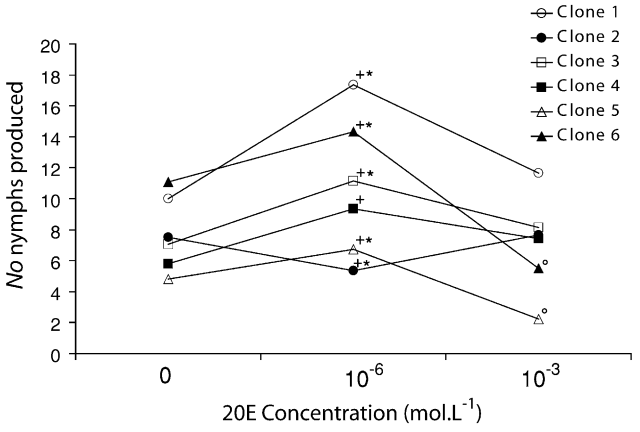


Fig. 1. Mean number of nymphs produced by each clone at each concentration of 20E. + corresponds to significant difference between $10^{-6}$ and 0 mol $L^{-1}$; * corresponds to significant difference between $10^{-6}$ and $10^{-3}$ mol $L^{-1}$; ° corresponds to significant difference between $10^{-3}$ and 0 mol $L^{-1}$. Tests were performed with the LSMeans option of the GenMod procedure of SAS (see methods). LSMeans are calculated from data for 15 adults per clone and per concentration.

1, 3 and 4 performed better at a 20E concentration of $10^{-6}$ mol $L^{-1}$ than at a concentration of 0 mol $L^{-1}$ ($P<0.001$ in all cases) and clones 1 and 3 performed better at a 20E concentration of $10^{-6}$ mol $L^{-1}$ than at a concentration of $10^{-3}$ mol $L^{-1}$ ($P<0.0001$ and $P=0.008$, respectively). No significant difference was observed between reproductive performances at 20E concentrations of 0 and $10^{-3}$ mol $L^{-1}$ for these clones ($P=0.179$, 0.290 and 0.089, respectively). Conversely, clone 2 performed less well at a 20E concentration of $10^{-6}$ than at concentrations of 0 and $10^{-3}$ mol $L^{-1}$ ($P=0.021$ and 0.013, respectively), with no difference detected between concentrations of 0 and $10^{-3}$ mol $L^{-1}$.

### 4.3. Choice experiment

The proportion of individuals on the control solution was found to be significantly affected by "clone" ($P<0.001$) but not by "concentration" or the "clone × concentration" interaction ($P>0.23$ in both cases). Thus, the proportion of individuals avoiding 20E differed between clones but we obtained no evidence to suggest that clones could distinguish between low and high concentrations of 20E.

Significant deviation from a 1:1 ratio of individuals on the two sides of the feeding cylinder was detected in three of twelve tests (Fig. 2). For clone 3, the proportion of individuals in the control zone was lower than the proportion of individuals on the $10^{-6}$ mol $L^{-1}$ solution ($P=0.002$). For clones 2 ($P=0.004$) and 6 ($P=0.030$), the proportion of individuals in the control zone was higher than the proportion of individuals on the $10^{-6}$ and $10^{-3}$ mol $L^{-1}$ solutions.

The proportion of individuals in the control zone in the choice experiment was positively correlated with the difference between the numbers of nymphs produced in the presence and absence of 20E ($r=0.63$, $P=0.03$; Fig. 3). Thus, the clones that preferred the control solution performed best on that solution. The three cases in which a significant deviation from a 1:1 ratio was detected in the choice experiment corresponded to clones displaying highly contrasted performances at each of the 20E concentrations in the no-choice experiment (see Fig. 1 and Table 1). The choice test was therefore probably not powerful enough to detect slight deviation from the 1:1 ratio.

## 5. Discussion

### 5.1. Genetic variability of the response of myzus persicae to 20E

The clones used in this study were collected immediately after a sexual reproduction event. The traits measured were thus measured on sexually differentiated clones, as shown by microsatellite analysis. Various significant differences in the responses to 20E were detected among the *M. persicae* clones used in this study. The reference concentration for comparisons was 0 mol $L^{-1}$. We found that $10^{-6}$ mol $L^{-1}$ increased or decreased the number of nymphs produced in the no-choice experiment, depending on the clone. The higher concentration of 20E ($10^{-3}$ mol $L^{-1}$) either had no effect or decreased the number of nymphs, depending on the clone tested. The choice experiment showed that the proportion of individuals avoiding the solution containing 20E also differed between clones. As clones were maintained in the laboratory for several months on the same

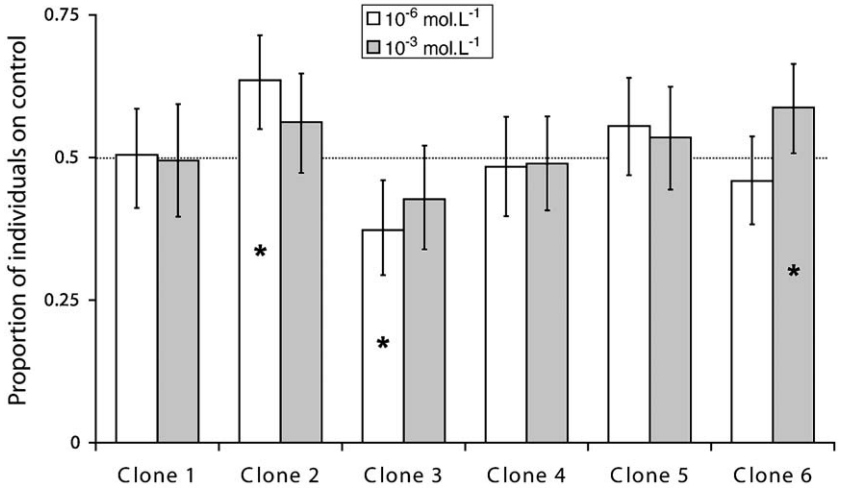


Fig. 2. Proportion of individuals, for each clone and each tested 20E concentration, feeding on the control solution (without 20E) in the choice experiment. * corresponds to significant deviation from a 1:1 ratio. Confidence intervals for these proportions were calculated using the modified Wald method, as described by (Agresti and Coull, 1998).

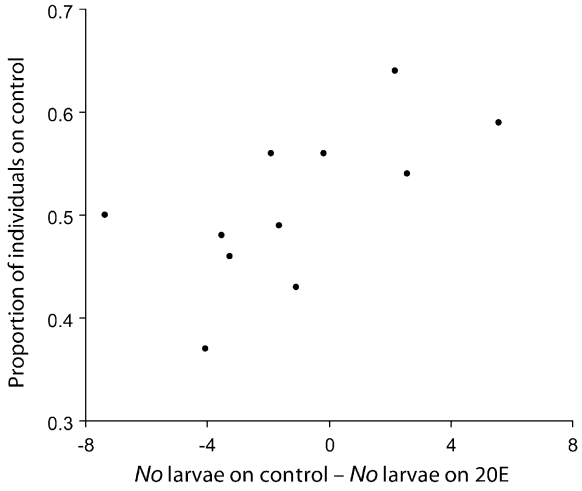


Fig. 3. Correlation between (1) the proportion of individuals found on the control solution (without 20E) in the choice experiment and (2) the difference between the mean number of nymphs produced by adults on the control solution and the mean number of nymphs produced by adults on a solution containing 20E ($10^{-6}$ or $10^{-3}$ mol $L^{-1}$). Pearson's correlation coefficient $r = 0.63$, $P = 0.03$.

host and were transferred simultaneously to the nutrient solution, the clonal variability observed here is extremely likely to arise from genetic variability (rather than from differences in maternal effects between clones).

### 5.2. Physiological or behavioural effects?

The positive correlation between the proportion of individuals in the control zone in the choice experiment and the difference between the numbers of nymphs produced in the presence and absence of 20E in the no-choice experiment suggests that (1) clones may have evolved the capacity to detect 20E and to select foods accordingly, so as to avoid the toxic effects of this molecule, or (2) the performance of clones in the no-choice experiment varies because 20E may have a deterrent or attractive effect and merely decreases or increases the food intake of individuals, without having toxic or endocrine disruption effects.

Deterrent and toxic/endocrine disruption effects of 20E have been reported in various types of insect larvae: abnormal development (Arnault and Sláma, 1986; Blackford and Dinan, 1997c; Kubo et al., 1983; Robbins et al., 1970), toxic effects on cells (Hu et al., 2004) and differential feeding responses (Marion-Poll and Descoins, 2002; Schmelz et al., 2002; Tanaka et al., 1994). None of these hypotheses can be excluded for *M. persicae*. Indeed, toxic effects of 20E on aphids, possibly including an impact on aphids' symbionts, may exist but our experiments are not conclusive to this regard. In addition, as the amount of solution consumed by each individual was not determined in the no-choice experiment, we cannot demonstrate a clear relationship between feeding behaviour and nymph production. Similarly, as the outcome of choice experiments was recorded after 24 h, we cannot exclude the possibility that aphids sense the presence of 20E through their taste receptors or via post-ingestion mechanisms (Glendinning, 1996).

One surprising result was the enhanced performance of aphids with a low concentration of 20E in their diet. Positive effects of ecdysteroids on insects were reported in *Bombyx mori* (Makka et al., 2002) on which 20E is used to synchronize larval development and silk production (Ninagi and Maruyama, 1996). Fragoyiannis et al. (1998) and Guntner et al. (1997) reported that an artificial diet containing the steroidal molecule glycoalkaloid α-chaconine increased the food intake and fecundity of *M. persicae*. However, little is known about the possible function of this ecdysteroid in aphids, and the only

published report concerns the identification of this molecule in the vetch aphid *Megoura viciae* Buck (Kulcsar et al., 1994). Whether this effect is specifically due to 20E or to a general stimulatory effect of low doses of a toxicant (hormesis) (Calabrese and Baldwin, 2001) remains an open question at this stage.

At the high 20E concentration, most of the aphid clones fed on the nutrient solution with no detectable detrimental effect on nymph production. Tolerance to this ecdysteroid probably results from aphids being able to metabolize and/or to excrete it. Some insects (Blackford et al., 1997; Modde et al., 1984; Zhang and Kubo, 1993), spiders (Connat et al., 1988) and mites (Diehl et al., 1985) have been shown to excrete phytoecdysteroids conjugated in their guts. Alternatively, 20E tolerance in *M. persicae* may result from the use by sucking homopterans of another ecdysteroid, makisterone A, as an endogenous hormone (Kelly et al., 1984). This would suggest that makisterone A is likely to have a stronger insecticidal effect than 20E.

### 5.3. Long-term selection pressures as a tool for managing pest species?

Our study focused on a particular set of phenotypic traits measured on adults, but the results strongly suggest that divergent long-term selection pressures exerted by host plants have maintained genetic variability in the response of *M. persicae* to 20E. As a result, responses to phytoecdysteroids are likely to be variable in nature populations, possibly even more so than in the sample of clones tested in this study. This genetic variability associated with the generally weak negative effect of 20E on clones implies that resistance to 20E would probably be rapidly selected in *M. persicae* if this molecule were used for pest control. The rapid selection of resistance genes would also be enhanced by the asexual reproduction phase of this species, which makes it possible to expand clonal populations, without recombination between genotypes with and without adaptation to 20E.

Ecdysteroid agonists and antagonists also require consideration as several insect species have been found to be sensitive to these molecules (Seth et al., 2004), in some cases even more so than to 20E (Blackford and Dinan, 1997b). The costs associated with resistance to ecdysteroids and their agonists/antagonists have not yet been investigated. However, the ecdysteroid-related sensory systems and metabolic pathways probably vary in existing genotypes, constituting a potential basis for the evolution of resistance. Mechanisms of resistance to currently used insecticides have evolved despite pleiotropic effects of the corresponding mutations, which are assumed to be associated with fitness costs in several insect species (see review in Coustau et al., 2000). Genetic support for the ecdysteroid-related pathways is therefore likely to evolve even more rapidly, as there may already be variation between individuals. Thus, it is essential to estimate the genetic variability of the responses of pest species to 20E and, more generally, to molecules that could potentially be used in pest management. Experimental tests of sensitivity are of potential value, provided that they are able to assess the responses of biologically and/or ecologically differentiated populations.

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