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ORIGINAL ARTICLE

# Evidence of active oviposition avoidance to systemically applied imidacloprid in the Colorado potato beetle

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**Abstract** Agricultural pests can develop behavioral resistance to insecticides by choosing to feed or oviposit on insecticide-free hosts. As young larvae have relatively low mobility, oviposition preferences from female adults may play a critical role in shaping the evolutionary trajectory of pest populations. While oviposition avoidance of insecticide-treated hosts was found in different agriculture pests, it remains unclear whether female adults actively choose to occupy insecticide-free hosts. To address this question, we investigated feeding and oviposition preferences between imidacloprid-treated and imidacloprid-free plants in the Colorado potato beetle, *Leptinotarsa decemlineata* Say, a major potato pest. We performed behavioral choice assays on two strains that differed in both fecundity and insecticide resistance. We found that one strain preferred to feed on the insecticide-free plants and that this preference is not innate. Meanwhile, the other strain chose plants for feeding and oviposition randomly. Further analyses of the moving patterns of the beetles suggested that the oviposition preference in the first strain is likely due to active learning.

**Key words** Colorado potato beetle; host preferences; imidacloprid; insecticide resistance; oviposition; pesticide avoidance

## Introduction

Rapid evolution of insecticide resistance threatens both global food supply and human health (Whalon *et al.*, 2008). Understanding and managing the evolution of insecticide resistance in pests requires integrated investigations on the underlying physiological, biochemical, and behavioral mechanisms. While significant progress has been made in identifying physiological and biochemical mechanisms (Feyereisen, 1995; Daborn *et al.*, 2002; Weill *et al.*, 2003), the extent to which behavior contributes to rapid evolution of insecticide resistance remains largely unclear. In nature, insecticides can be heterogeneously distributed within a plant and between plants resulting in plenty of niches with no or low levels of insecticide (Olson *et al.*, 2004; Huseth *et al.*, 2014). Hence, insects can use avoidance behavior to feed or live on insecticide-free or low-insecticide hosts over high-insecticide hosts and thus survive insecticides. Selection could therefore theoretically favor such behavioral traits (Muirhead-Thomson, 1960; Georghiou, 1972; Lockwood *et al.*, 1984; Khodaverdi *et al.*, 2016). Because young larvae have limited ability to move and are more susceptible to food quality, the oviposition choice of adult females plays a critical role in determining the fitness of larvae. As many adult insects feed on the host before oviposition, it is possible that female adults can detect the insecticide and its metabolites in the host and actively choose to lay eggs on hosts with no or low levels of insecticide.

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However, this has never been experimentally verified before, despite the existence of oviposition avoidance behavior in several insects (Tomé *et al.*, 2013; Nansen *et al.*, 2016; Zhao *et al.*, 2016; Andreazza *et al.*, 2018).

The Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) is currently considered a major pest worldwide (Alyokhin *et al.*, 2022). It can cause complete defoliation of potato plants if left uncontrolled (Hare, 1980). The beetle is thought to be native to Mexico where it fed on *Solanum rostratum* (buffalobur) before expanding its host range to *Solanum tuberosum* (potato) (Casagrande, 1987; Piiroinen *et al.*, 2013; Izzo *et al.*, 2018). Pest populations of the beetle emerged in North America (Izzo *et al.*, 2018) and later spread to Europe, and parts of Asia. Currently, over 300 resistance cases have been reported in CPB populations globally spanning over 54 different insecticides including the neonicotinoid imidacloprid, commonly used in agriculture (Mota-Sanchez & Wise, 2022). Imidacloprid is an agonist of the insect nicotinic acetylcholine receptor that can act upon direct contact or ingestion causing paralysis and eventually death (Schroeder & Flattum, 1984; Bai *et al.*, 1991). Increased metabolism, target site insensitivity, reduced insecticide penetration, and enhanced excretion are some of the known mechanisms of resistance in CPB (Alyokhin *et al.*, 2008). Unlike these physiological and biochemical processes, behavioral resistance is difficult to unequivocally prove owing to the complex nature of behavior, lack of knowledge of behavior prior to insecticide exposure, and difficulty in confirming that the behavior does increase the possibility of resistance (Zalucki & Furlong, 2017). Thus, the extent to which behavior contributes to the evolution of insecticide resistance in CPB remains largely unknown. While some studies did observe behavioral differences between resistant and susceptible CPB strains (Hoy & Head, 1995; Alyokhin & Ferro, 1999b), it is still unclear whether CPB can preferentially inhabit and lay eggs on insecticide-free or low-insecticide hosts given a choice. Establishing such behavioral preferences is an essential first step in tackling this lesser known but potentially crucial field of insecticide resistance studies.

CPB often prefers domesticated potato (*S. tuberosum*) plants as its hosts, both for feeding and oviposition, although some contradictory evidence from different geographical populations exist (Hsiao & Fraenkel, 1968; Bongers, 1970; Hsiao, 1978; Hiiesaar *et al.*, 2020). Host finding is facilitated by visual and olfactory cues from a distance (De Wilde, 1958; Mitchell, 1988; Landolt *et al.*, 1999; Dickens, 2000). Once they are within a close range, the larvae and beetles are attracted to volatiles associated with *Solanum* plants (Visser & Nielsen, 1977; Dickens, 2000). After finding a host, specific orientation and a series of sequential feeding behaviors that follow upon contact determine its acceptance or rejection (De Wilde, 1958; Harrison, 1987; Visser, 1988; Mitchell & Low, 1994). During their elaborate biting and sampling routine, CPB adults and larvae use their sensitive gustatory cells to taste the compounds in a given host to determine whether it is suitable (Mitchell, 1974; Harrison, 1987; Mitchell & McCashin, 1994; Zhang & Mitchell, 1997). Experience or learning influences the host preference in CPB (Visser & Thiery, 1986) as it does in many insects like flies, moths and butterflies (Swihart & Swihart, 1970; Saxena & Schoonhoven, 1982; Jaenike, 1986).

Feeding preferences of the larvae and adults are generally similar, although the host plant range of the larvae may be more restricted (Chin, 1950). Once a host is accepted, continued feeding, mating and oviposition subsequently follow. Oviposition preference is generally thought to be consistent with the feeding preference because the gravid females fly fewer times than unmated females (Alyokhin & Ferro, 1999a), although there is some evidence for discrepancies between feeding and oviposition preferences (Hsiao & Fraenkel, 1968; Bongers, 1970). However, all of feeding and oviposition preferences of CPB were measured among different plant species. To our knowledge, no study has examined the feeding and oviposition preference between insecticide-treated and untreated plants in CPB.

Here, we investigated CPB's behavioral response to systemically applied imidacloprid, both in terms of feeding and oviposition choices. To this end, we first screened five different CPB strains and quantified the feeding and oviposition preferences of two of them. We aimed to address the following two main questions: (1) Do CPB larvae and/or adults actively avoid feeding on insecticide-treated plants? (2) Do adult females actively avoid insecticide-treated plants for oviposition? Answers to these questions will sharpen our understanding of behavioral resistance in a major agricultural pest, which might aid the successful implementation of integrated pest management strategies in the future.

## Materials and methods

### Plants, insects, and insecticide

Five-week-old *S. tuberosum* plants (Annabelle variety, seed potatoes purchased from TOLLS Kartoffelhandel GmbH & Co. KG, Willich, Germany) were used for rearing the beetles, and for the experiments. Annabelle variety was chosen due to its relatively short growth time, sufficient leaf mass and ready availability in the region.

The growing of plants, rearing of the beetles and experiments were all carried out in a greenhouse, under a long day photoperiod (16 h : 8 h / L : D) and a temperature of 24 °C. Nine to twelve tubers were planted in trays of size 39 cm × 29 cm × 7 cm containing peat soil (Baumschul & Stauden Type, purchased from Balster Einheitserdewerk GmbH, Fröndenberg, Germany). Once the tubers were planted, the trays were watered every three to five d. Additionally, an NPK fertilizer (Compo Expert Hakaphos Blau, purchased from Meyer-shop, Rellingen, Germany) was mixed into the water once every three weeks before watering.

Colorado potato beetles were obtained from Dr. Ralf Nauen's lab, Bayer AG (Monheim, Germany). The five strains used in the study were collected by the supplier and group from different parts in Europe at different points in time (Mehlhorn *et al.*, 2020). They are, namely, D01 (Germany, 2002), E01 (Spain, 2014), E02 (Spain, 2017), E06 (Spain, 2012), and U01 (Ukraine, 2012). All insects had been reared under insecticide-free conditions for at least 10 generations in 85 cm × 45 cm × 55 cm sized insect cages. During each generation, eggs were collected regularly using wet brushes and stored at 10 °C for synchronizing hatching times. Afterward they were moved back into the greenhouse. Water was sprayed on top of the eggs to prevent drying. After 5 d, when the larvae hatched, they were introduced into cages with sufficient potato plants where they were allowed to feed on the leaves and develop. To keep the soil dry enough for pupation, the watering of the plants was stopped once the larvae reached the final stage of development. The larvae were identified to be in the late fourth instar stage by visually observing the larger size and the color change from red to orange. Then, the larvae were allowed to fall into the trays and pupate within the soil. When the adults emerged 11 d later, they were supplied with fresh potato plants.

The neonicotinoid insecticide imidacloprid was used in our experiments in the form of water dispersible granules available commercially by the name Confidor WG 70 (70 g/kg imidacloprid, Bayer AG, Monheim, Germany). The insecticide granules were mixed in water and applied to the soil. The exact concentrations used for each experiment and the reasons for choosing the concentrations are described in the following sections.

### *Toxicity assays with larvae*

Toxicity assays were performed with 2-d-old 1st instar larvae following the IRAC Susceptibility Test Method 029 (IRAC Methods Working Group, 2013). The larvae were placed on a Petri dish lined with filter paper. For each of the five strains (D01, E01, E02, E06, U01), aqueous insecticide solutions of four different concentrations calculated by weight (15.6 ppm, 62.5 ppm, 250 ppm, 1000 ppm) and a control (water without insecticide) were tested. For each concentration, six replicates were tested, and each replicate contained five larvae. We applied 1 $\mu$L of the solution for the respective treatment using a micropipette (control or one of the 5 insecticide solutions) on the top of each larva. The larvae were supplied with enough leaves to feed on throughout the duration of the assay. Mortality was recorded after 48 h. The reasons for choosing the concentrations mentioned above are available in the Supporting Information Section 1.

The concentration for the subsequent experiments was determined from a combination of the toxicity assays and pretests. A dose that is too high can kill many individuals before a choice can be detected, thus confounding behavioral results with the physiological effects of insecticide consumption. But a concentration that is too low can prevent the discrimination of insecticide-treated and insecticide-free plants. This can in turn obscure preferences that might be detected at higher concentrations. For this reason, 2 ppm, which corresponded to the concentration that kills 25% larvae of the most sensitive D01 strain, was used in the choice assays. This concentration reflected a good sublethal concentration in our opinion because of the relatively low mortality.

Insecticide quantification was performed to monitor the amount of imidacloprid in the leaves of potato plants after watering their soil with an aqueous solution of 2 ppm imidacloprid (insecticide-treated) or with pure water (control). The experiments were conducted with 5-week-old plants and five replicates were collected 0, 1, 2, 3, 5, 7, 10, and 14 d following the treatment. Samples were collected by cutting leaf discs with a 1.75 cm diameter cork borer from the leaves at the 3rd, 4th, or 5th position (from the top) of insecticide-treated and control plants. The leaf discs were snap frozen in liquid nitrogen and thoroughly homogenized using a Retsch ball mill. Approximately 10−15 mg (fresh weight or FW) of finely ground plant material was extracted with 1 mL of 70% MeOH with 1% formic acid (Fisher Chemical) as extraction buffer supplemented with 10 ng/mL $D_4$-imidacloprid (imidacloprid deuterated four times, Sigma-Aldrich) as an internal standard. After thoroughly shaking, the samples were incubated for 30 min on ice, centrifuged at 16 900× *g* for 20 min at 4 °C, and subsequently the supernatant was collected. The pellet was reextracted with 0.5 mL extraction buffer without internal standard, by another round of shaking, centrifugation and collecting the supernatant. Both supernatants were combined and centrifuged again to remove the remaining particles before analysis.

Chromatography was performed on a Nexera X3 UHPLC system (Shimadzu). For separation, a Zorbax RRHD Eclipse XDB C18 column (1.8 $\mu$m, 3 × 50 mm; Agilent) was used. The mobile phase comprised solvent A (water, 0.1% (v/v) formic acid, 0.05% (v/v) acetonitrile) and solvent B (methanol) with the following elution profile: 0 min, 10% B; 0.5 min, 10% B; 1 min, 25% B; 4 min, 30% B; 4.5 min, 100% B; 5.5 min, 100% B; 6 min, 10% B; 7 min, 10% B; with a flow rate of 0.5 mL/min. The column temperature was maintained at 42 °C. The chromatography was coupled to an LCMS-8060 mass spectrometer (Shimadzu) with an ESI source. The instrument was operated in positive ionization mode multi-reaction-monitoring modus to analyze the parent ion to the product ion transitions of the analytes (Table S1). The following source parameters were used: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; drying gas flow, 10 L/min; interface temperature, 300 °C; DL temperature, 250 °C; heat block temperature, 400 °C. The CID gas flow was set to 270 kPa and the resolution for Q1 and Q3 to unit resolution.

Based on the determined insecticide levels, for all subsequent experiments, the plants were treated 2 d before the introduction of the insects to ensure the insecticide had reached the leaves (Fig. S1).

### *Choice assays (feeding and oviposition)*

The choice assays were performed with two strains, namely, the D01 and E06 strains. For the insecticide treatment, a low concentration of 2 ppm was chosen in order to eliminate the confounding effects of mortality on behavioral choices. The insecticide-treated (*T*) plants were treated with 1 L of an aqueous insecticide solution containing 2 ppm imidacloprid 2 d before the experiment. The insecticide-free (*C*) plants were treated with 1 L of a control solution (water without insecticide) 2 d before the experiment.

Behavior assays with larvae were performed in a Petri dish setup. The innate feeding preference, that is, a preference that preceded any experience with a host, was tested using freshly hatched naïve larvae. To assess the effects of prefeeding, larvae that had been fed for 24 h with leaf cuttings from either *C* or *T* plants (leaf positions 3–5) were used. For each assay, the preference of 60 individuals was tested for the D01 and E06 strains, respectively. For each individual choice test, a single larva was placed in a Petri dish arena with two choices of leaf discs (one disc per choice). The leaf discs were cut using cork-borers of 1.75 cm diameter from *C* and *T* plants 2 d after treatment. The Petri dishes were lined with filter paper and lightly sprayed with water to slow down drying up of the leaf discs. The two types of leaf discs were set up on opposite sides and the Petri dishes themselves were turned around 0–360° randomly, allowing for cancellation of any external effects like light or temperature. The larva was picked up with a wet paintbrush and left at the center of the Petri dish arena. The first choice was defined as the leaf disc from which the larva started eating. During 4 h of continuous observation, the first, and subsequent choices, if any, were noted.

Behavioral assays with adults were performed using a cage setup. The adults used in the experiment had freshly emerged from the soil. Their development during the larval stage was on insecticide-free plants. The feeding and oviposition choice behavior were closely monitored for adults of both of the strains in a cage setup inside which an insecticide-free (*C*) tray and an insecticide-treated (*T*) tray of potato plants were present. Two days after treatment, the trays were arranged one after the other inside the cages and the order of the trays was randomized between replicates to avoid any external effects. Eight male and eight female adults were introduced into the center of each cage, on the cage floor, in between the two choices of plants with a 15-min interval between the sexes (order of introduction randomized between replicates). For each strain, 10 replicates were used. The feeding behavior of the adults was closely observed for 30 min and the number of beetles that started to feed from both *C* and *T* plants was recorded for both sexes. In the following days, the number of beetles in each of the trays was recorded twice daily to track the beetle movement patterns. The eggs were collected daily to track the oviposition preferences. During the daily egg collection, special care was taken to ensure that leaves holding the eggs were cut just around the egg masses in order to reduce the amount of leaf material unnecessarily removed from the plants. In addition to the number of eggs, the number of egg masses was also recorded. After 10 d, the total number of eggs and the number of egg masses were calculated. The net mortality (total adults dead at the end of the experiment) and the percentage of leaf damage was recorded at the end of the experiment after 10 d, based on counts and visual inspection respectively. In detail, the percentage of leaf damage was roughly estimated by visually inspecting the trays and recording the damage. This method was used to quantify defoliation mainly due to the lack of image capturing and processing systems that can accurately estimate defoliation in a heterogeneous, three-dimensional environment. The blinded nature of the choice assay eliminates any bias in this method. Furthermore, the observer being the same for all trays removes any differences in classification standards between different observers. The choice assays with adults were later repeated with the E01 strain instead of D01. Details for this experiment are available in the Supporting Information Section 2.

### *Data analysis*

All analyses were done using the "stats" package (and functions therein) implemented in R Core Team (2020). For estimating the lowest concentrations of imidacloprid in the insecticide-water solution required to kill 50% (LC50) and 90% (LC90), respectively, we followed the method described by Crawley (2014, pp. 635−636). Briefly, our response variable is the counts of "dead" or "alive" larvae, from which we performed a binomial generalized linear model (GLM) and a logistic regression to predict LC50 and LC90. The standard error of the mean was calculated using the dose.p function in the MASS package (Crawley, 2014). This function takes a vector of concentrations (0 ppm, 15.62 ppm, 62.5 ppm, 250 ppm, 1000 ppm) as input. The 95% confidence intervals were calculated using the Wald statistic.

The choice preference was quantified using a choice index (*CI*), which was calculated as: $CI = \frac{T-C}{T+C}$, where $T$ and $C$ represent the "choice" for insecticide-treated plants and control plants, respectively (Takakura, 2009; Musa *et al.*, 2017; Ratnaweera *et al.*, 2020). "Choice" is used here as a general term to denote the number of larvae or the number of beetles that choose $T$ or $C$, or the number of eggs that were laid on $T$ or $C$. This index gives values above 0 if $T$ is preferred and values below 0 if $C$ is preferred. The advantage of using the choice index rather than the raw count data is that the choice index normalizes the differences in counts between the groups. Thus, by using the choice index, the response variable is set within the same scale ($[-1, +1]$) for both strains irrespective of any intrinsic differences in fecundity.

### *Feeding choice*

For larvae, the response variable to assess the effects of prefeeding treatments and the strain on the feeding choice was the *CI* and a GLM was fitted to the data assuming a binomial distribution. To further check if the feeding preference of the larvae is significantly different from a random choice, the choice was denoted as either 0 (control) or 1 (insecticide-treated) and a binomial test to compare the two proportions was used for all treatment groups.

For adults, the first feeding choice data constituted of the counts of beetles on control or insecticide-treated plants. From these counts, *CI* was calculated and used as the response variable to analyze the effects of strain for which a GLM was fitted to the data, assuming a normal distribution. For the beetle movement pattern data, the *CI* was modeled using Linear Mixed Models with the strain as a fixed effect and the day as a random effect, because of the repeated measures nature of the design. For the leaf damage, the response variable was the percentage leaf damage, which ranged from 0% to 100% (in categories of 5% and 20%). To analyze the effects of strain and treatment on leaf damage, a GLM was fitted assuming a negative binomial distribution. We chose a negative binomial GLM because the variance was much larger than the mean.

### *Oviposition choice*

For oviposition choice data, the response variables were the total number of eggs and the total number of egg masses. To analyze the effects of strain and treatment on oviposition, a GLM was fitted assuming a negative binomial distribution. We chose a negative binomial GLM because the counts were large, and the variance was much larger than the mean. For the oviposition pattern data, the choice index was modeled using Linear Mixed Models with the strain as a fixed effect and the day as a random effect. *P*-values were determined using Analysis of Variance (ANOVA).

## Results

### *Toxicity assays using 1st instar larvae*

To determine the levels of susceptibility of the five different strains of CPB, we performed dose-response experiments using the first instar larvae (see Methods section). Among all five strains, E06 strain is the most resistant one, which is 25 times more resistant to imidacloprid than the D01 strain (Fig. 1). The lowest average concentrations to kill 50% (LC50) and 90% (LC90) of the larvae are shown for all five strains in Table S2.

### *Behavioral avoidance in larvae*

To examine behavioral avoidance at the larval stage, we performed choice assays in Petri dishes using the D01 and E06 strains. 45% of naïve D01 larvae and 39% of naïve E06 larvae chose the control plants. Thus, the

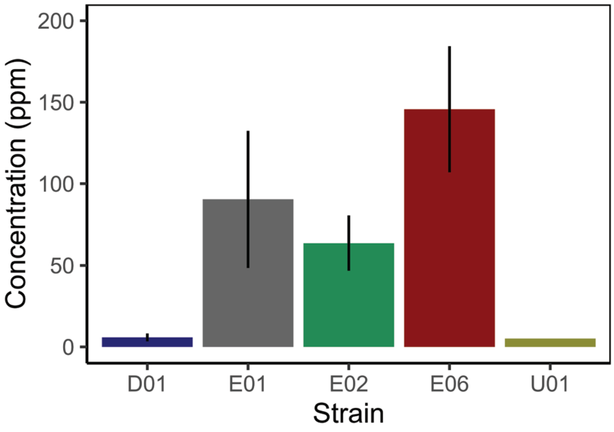

**Fig. 1** Levels of imidacloprid tolerance differ among five studied Colorado potato beetle strains. LC50 values for 2-d-old larvae of the five strains when insecticide-water solution is applied topically. The concentration of imidacloprid in the insecticide-water solution is on the *Y*-axis in ppm. The colors represent the five different strains as indicated on the *X*-axis. Error bars represent 95% confidence intervals.

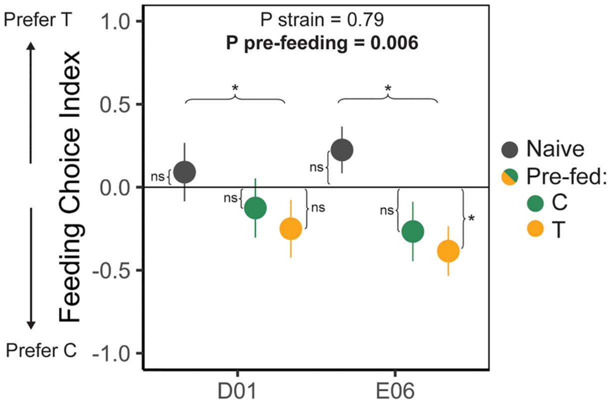

**Fig. 2** Larvae do not show any innate feeding preference, but prefeeding affects choice. Choice index is plotted for both the D01 strain and the E06 strain. The first choice was recorded within 4 h after the larvae were introduced into the Petri dishes. The solid horizontal line corresponds to a choice index of 0. Bars represent the standard error of the mean. The colors represent the prefeeding treatment [Gray = no prefeeding, Green = prefed with control leaves (imidacloprid–), and Orange = prefed with insecticide-treated leaves (imidacloprid+)]. The horizontal brackets and asterisks represent the significant difference of the prefeeding treatments (both Control and Treated) from the no prefeeding treatment. Preferences not significantly different from $CI = 0$ : D01, naïve ($P = 0.73$), E06, naïve ($P = 0.15$), D01, prefed on $C$ ($P = 0.6$), D01, prefed on $T$ ($P = 0.22$), E06, prefed on $C$ ($P = 0.2$). Significantly different from $CI = 0$ : E06, prefed on $T$ ($P = 0.02$) (Binomial tests). ns, not significant; $^*P < 0.05$.

freshly hatched naïve larvae of either strain did not show a significant preference between control and insecticide-treated plants, suggesting negligible innate preference of CPB larvae (Fig. 2, gray circles). However, 1 d of prefeeding on insecticide-free or insecticide-treated potato plants statistically affected the choice of the larvae of both strains (Fig. 2, green circles: prefed on control plants; orange circles: prefed on insecticide-treated plants). Among the larvae that were prefed on control plants, 56% of D01 larvae and 63% of E06 larvae chose control plants. Among the larvae that were prefed on treated plants, 62% of D01 larvae and 69% of E06 larvae chose control plants. The effect is small as the choice index after feeding remains close to 0. Strain did not have an effect on larval feeding preference ($P = 0.79$).

During the observation period of 4 h, no larvae from either strain switched positions from the first chosen disk to the other in any of the treatments. Additionally, first choices were not influenced by the lethal effects of the insecticide since all the larvae that died during the assay died before making a choice (Table S3).

### *Behavioral avoidance in adults*

We further measured behavioral avoidance of the two strains at the adult stage using choice assays. When control and insecticide-treated plants were present in the same cage, the first choice (within 30 min) appears to be random. The strain type did not have an effect on the choice ($P = 0.81$, Fig. 3A). No differences were found between males and females ($P = 0.38$).

Detailed observations on the presence of adults on control and insecticide-treated plants revealed interesting movements and feeding patterns of the two strains. The movement patterns for both strains were significantly different from each other ($P < 0.001$). Furthermore, we tested whether each strain was different from a random choice line ($CI = 0$). We found that the pattern for D01 was significantly different from $CI = 0$ showing a preference for control plants ($P < 0.001$), whereas E06 did not show any significant difference from $CI = 0$ ($P = 0.67$). When looking at the movement on a day-by-day basis, for both strains, there were an equal number of adults on both of the plant choices at the start. From day 4, the D01 strain gradually moved toward controls plants, while the E06 still did not show clear host preference. The strong preference for control plants in the D01 strain continued until the 7th day (Fig. 4A). As a consequence, the D01 strain consumed significantly more from the control plants while the E06 strain showed a tendency in the

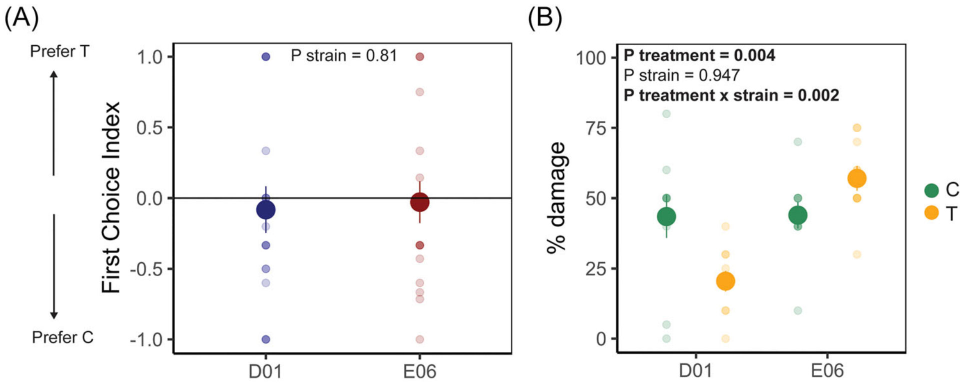

**Fig. 3** Adults do not show any innate feeding preference, but D01 adults consume more control leaves over time. (A) First choices of adult beetles within 30 min. Choice index graphs showing the preference of beetles in the bioassay with intact plants. The beetles were counted within the first 30 min of being introduced into the cages. The solid horizontal line corresponds to a choice index of 0, meaning that the numbers of beetles on the two choices of plants are equal. The solid dots represent the average number of beetles, and the faded dots show the value for each replicate. Bars represent standard error of the mean. The colors represent the two strains (Blue = D01 and Red = E06). Preferences not significantly different from $CI = 0$ : D01 ($P = 0.53$), E06 ($P = 0.49$) (*t*-tests). (B) Percentage of leaf damage from intact plants after 10 d. The mean percentage of leaves consumed in the choice assay with adult potato beetles is plotted for both the D01 strain and the E06 strain. Strains are shown on the *X*-axis. The solid dots represent the average consumption, and the faded dots show the value for each replicate. Error bars represent the standard error of the mean. The colors represent the two choices of plants (Green = Control and Orange = Treated).

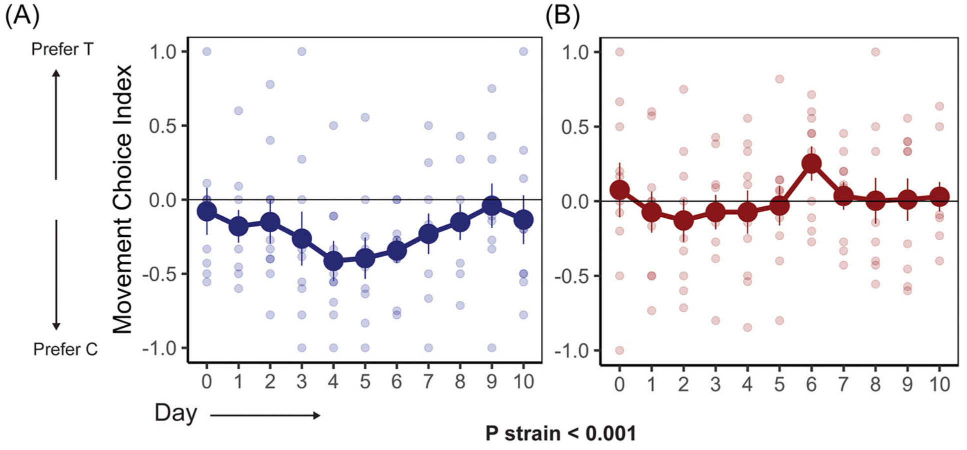

**Fig. 4** Movement pattern of the two strains (A = D01 and B = E06). The average number of beetles found on insecticide-treated (*T*) or insecticide-free (*C*) plants every day for 10 d is plotted here in the form of a movement choice index. The solid dots represent the average number of beetles, and the faded dots show the value for each replicate. The line segment connecting the solid dots shows the movement pattern. Error bars represent the standard error of the mean. The colors represent the two strains (Blue = D01 and Red = E06).

opposite direction (Fig. 3B). While there was no significant preference was found for the E06 strain, the interaction term in the model was statistically significant, proving that the preference of the E06 strain is the reverse of the preference of the D01 strain. From day 7, the number of D01 beetles on either plant is not different. This increased movement toward the treated plants is most likely due to reduced eatable leaf material left on the control plant.

Based on the eggs collected from each host plant, we calculated the oviposition choice index for female adults. The daily oviposition record showed that the D01 strain started laying eggs 2 d later than the E06 strain and exclusively laid eggs on control plants in the first two

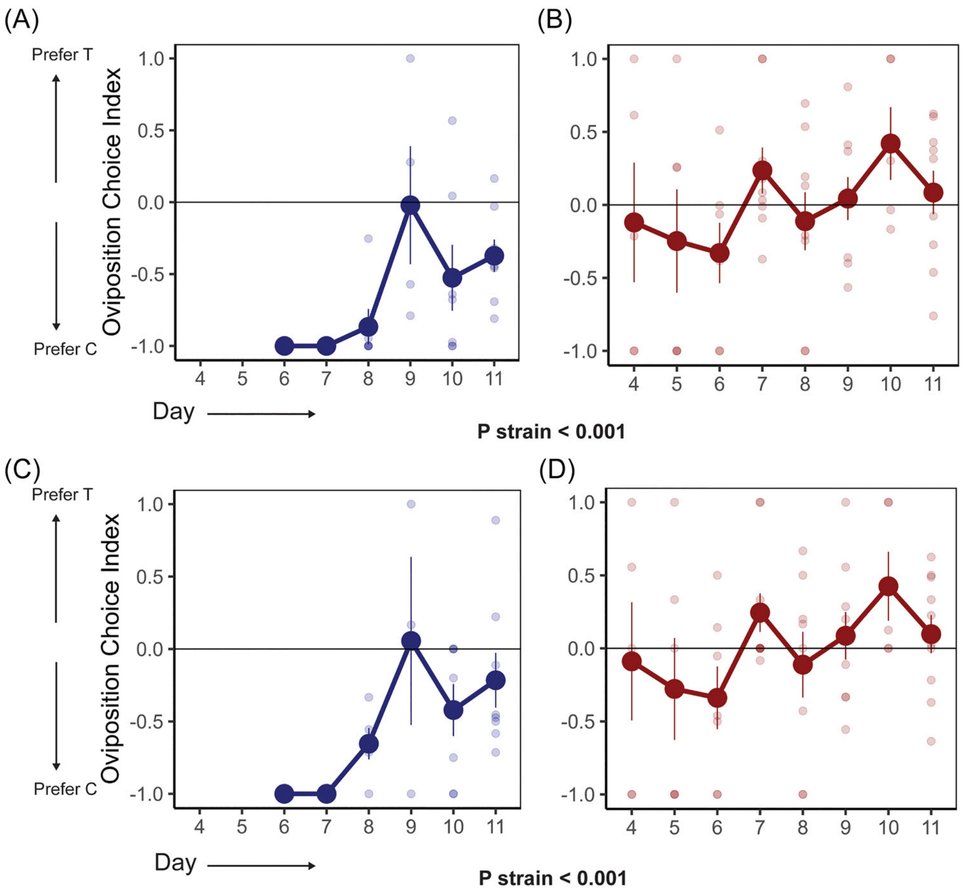

**Fig. 5** Oviposition choice of the two strains (A & C = D01 and B & D = E06). The average numbers of eggs (top panels, A & B) and egg masses (bottom panels, C & D) found on each of the plants every day for the duration of the experiment are plotted here in the form of an oviposition choice index. The solid dots represent the average number of eggs/egg masses, and the faded dots show the value for each replicate. The line segment connecting the solid dots shows the oviposition preference pattern. Error bars represent the standard error of the mean. The colors represent the two strains (Blue = D01 and Red = E06).

oviposition days (Fig. 5A–D). Over the whole duration of the experiment, the E06 strain laid about 3 times more eggs in total than the D01 strain (Fig. 6A). While the D01 strain laid 73.4% of the eggs on control plants, no statistically significant preference was found for E06 strain (Fig. 6A, B). However, the interaction term in the oviposition pattern model was statistically significant, showing that the E06 strain might prefer to oviposit on toxic plants.

## Discussion

Analyzing the levels of behavioral resistance is critical for understanding the evolution of insecticide resistance and host plant adaptation in general. Here, we showed that a strain of CPB adults showed clear behavioral avoidance, both in terms of feeding and oviposition choices, of insecticide-treated host plants. Additionally, we showed that these preferences are likely active choices made after experiencing insecticide-free and insecticide-treated plants.

A key factor to consider for behavioral preferences and learning is the concentration at which the insects are tested. Based on toxicity assays and choice assay pretests, we chose a sublethal concentration to be used in the choice experiments. Sublethal concentrations are prevalent in nature and can affect behavioral responses (Haynes, 1988; Olson *et al.*, 2004; Huseth *et al.*, 2014; Müller, 2018). For example, lethal and sublethal concentrations of neonicotinoids are known to affect learning and memory in honeybees, bumblebees and other pollinators (Desneux *et al.*, 2007; Tasman *et al.*, 2021).

We found some similarities and differences between larvae and adults in the behavioral avoidance of imida-

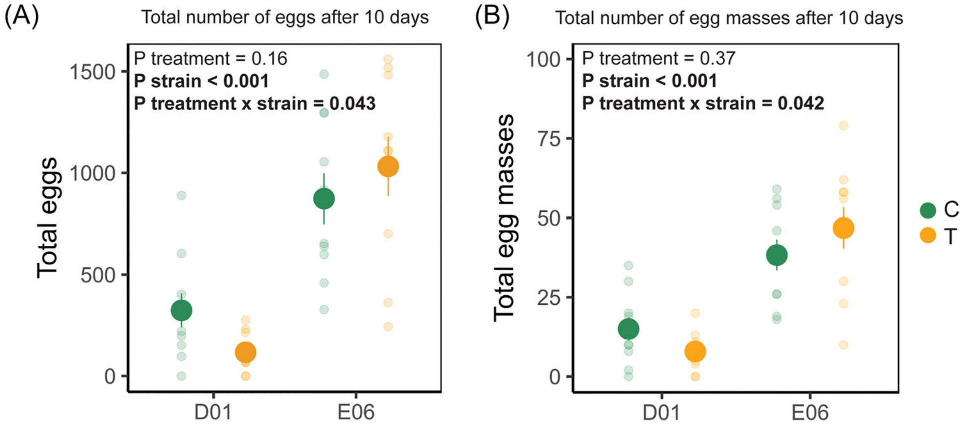

**Fig. 6** D01 strain lays more eggs on control plants than insecticide-treated plants. Total number of eggs and egg masses per replicate are plotted for both the D01 strain and the E06 strain as shown on the *X*-axis. The solid dots represent the average number of eggs, and the faded dots show the value for each replicate. Error bars represent the standard error of the mean. The colors represent the two choices of plants (Green = Control and Orange = Treated).

cloprid. Both naïve larvae and adults show no preference in their first choice. However, prefeeding for a day statistically increased the preference for control plants. The effect is small, as the choice index after prefeeding is still very close to 0. In addition, we do not consider this an active choice because they do not feed from both of the leaf discs before deciding to accept one. This suggests that active avoidance behavior probably appears in the later life stages of the beetles. Another difference is that in the case of larvae the strain does not have an effect in the choices.

The adult movement pattern data shows that the D01 strain prefers to feed on the control plants. For such a preference to manifest, the beetles must detect differences between hosts which can be facilitated primarily by olfactory and/or gustatory cues (Thorsteinson, 1960; Harrison, 1987). On the one hand, while olfactory cues, such as plant volatiles, play an important role in the host and mate finding behavior of the potato beetles (Visser & Nielsen, 1977; Dickens, 2000; Haber & Weber, 2022), it is unlikely that they play a significant role here for two reasons. First, it could be that the volatiles emitted by potato plants are not significantly affected by soil treatment of imidacloprid. We did not find any studies showing that application of imidacloprid via soil affects potato volatiles, although lowered levels of green leaf volatile emission upon spraying imidacloprid was previously reported in tea plants (Zhou *et al.*, 2019). In addition, the distance between control and insecticide-treated plants as well as the size of the cages are small, which reduces the likelihood of detecting different volatiles for CPB. Second, the first feeding choice is random both in the case of larvae and adults, which could indicate that there are no detectable differences in volatiles between insecticide-free and insecticide-treated plants inside our test cages. On the other hand, CPB are known to have sensitive galeal gustatory cells that respond distinctly to potato leaf alcohols, sugars, amino acids, and their specific combinations (Mitchell, 1974; Mitchell & McCashin, 1994). While the effect of systemic uptake of imidacloprid on the concentration of amino acids and sugars in potato leaves is a possible reason, another likely mechanism is the detection of insecticide itself and/or its metabolites via the gustatory system. Further electrophysiological and behavioral studies on the effects of insecticides on gustatory responses are needed to test this hypothesis.

Learning, which influences the host preference in many insects (Swihart & Swihart, 1970; Saxena & Schoonhoven, 1982; Jaenike, 1986), likely has contributed to the observed feeding and oviposition avoidance in the D01 strain. This can be seen from the gradual increase of preference to control plants after 3–4 d in the choice experiment with adults (Fig. 4). The preference disappeared after 7–8 d. The trays were heavily defoliated after 10 d with almost 50% of insecticide-free plants defoliated on average (Fig. 3B). This suggests that the disappearance of the preference on the later days was likely due to the low availability of plant material and increased competition. Similar learning effects are also seen in the oviposition pattern. The D01 strain started laying eggs on day 6 and nearly all eggs were on control plants in the initial 3 d. Aside from learning, the higher proportion of eggs found on control plants can also be due to the physiological effect of consuming insecticide-treated plants, which can reduce or delay the number of eggs laid by the beetles (Alyokhin & Ferro, 1999b). However, we think this is less likely given the correlated changes in their movement (Fig. 4) and oviposition choice patterns (Fig. 5).

Oviposition choice of the female adults can directly affect the survival of the less motile young larvae of the next generation. Hence, it is vital that the female chooses a site that can provide sufficient resources to ensure the survival of the offspring. The daily removal of eggs could have affected the movement and/or oviposition patterns due to females recognizing the absence of eggs. However, it has been shown that the absence of eggs does not change feeding patterns (Pelletier, 1995). We found that the D01 strain showed a strong preference for the insecticide-free plants and that the E06 strain did not show any preference. Interestingly, a similar pattern was observed in another susceptible strain E01 when compared with E06, suggesting that the avoidance behavior is a reproducible trait in CPB (Fig. S2−S5). While it is tempting to associate avoidance behavior with the levels of imidacloprid susceptibility, our experimental design cannot determine the relationships between these traits. Although susceptibility to insecticide could act as a strong selection pressure in the field and influence traits that affect both feeding and oviposition preferences, future studies that deploy quantitative genetic approaches should be performed to identify the underlying genetic architectures and associations between insecticide susceptibility and behavioral avoidance in CPB. Furthermore, our results could be used as a basis for investigating the contribution of avoidance behavior to the rapid global invasion of this pest.

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## Disclosure

RN is employed by the inventor and manufacturer of imidacloprid, Bayer CropScience.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1**. Imidacloprid quantification in potato leaves following soil application of Confidor.

**Fig. S2**. The movement pattern of the A. E01 and the B. E06 strains.

**Fig. S3**. Oviposition choice of the A. E01 and the B. E06 strains.

**Fig. S4**. E01 strain lays more eggs on control plants than on insecticide-treated plants.

**Fig. S5**. Imidacloprid quantification in potato leaves following soil application of technical grade imidacloprid.

**Table S1**. Multi-reaction-monitoring-settings and retention time for LC-MS-based imidacloprid quantification.

**Table S2**. LC50 and LC90 values along with the Standard Error of the Mean (SEM) and Resistance Ratio (RR) for 5 strains of potato beetle larvae from the toxicity assay by topical application.

**Table S3**. Larvae that died before making a choice in the feeding choice assay.

**Section 1**. On choosing the concentrations for toxicity assays.

**Section 2**. Choice assays with adults (repetition).