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Lethal and sublethal effects of imidacloprid, after chronic exposure, on the insect model *Drosophila melanogaster*

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ABSTRACT

Neonicotinoids are subjected to vigilance because of environmental contaminations and deleterious effects on bees. Imidacloprid (IMI) is one of the most representative insecticides of this family. At chronic exposure, concentration-effect relationships are non linear. An insect model should allow a better description of this toxicity. We compared the lethal concentration 50% (LC50) of IMI for a *Drosophila*-field strain, after acute and chronic exposure. Relative to the acute LC50, the chronic LC50 was lowered by a factor of 29 for males (1.3 mM/45 µM), 52 for larvae (157 µM/3µM) and more than 172 for females (>3.1mM/18µM). Chronic exposure also revealed significant lethal and sublethal effects, at concentrations 3-5 orders of magnitude lower than the chronic LC50. Mean mortalities reached 28% (at 3.91 nM) and 27% (at 39.1 nM) for females and males, respectively. Fecundity decreased of 16% at 1.96 nM. Mating increased of 30% at 0.391 nM. The LOEC (Lowest Observed Effect Concentration: 0.391 nM) was 46000 times lower than the chronic LC50 for males; it was 115000 times lower than the chronic LC50 for females. This study illuminates effects that neonicotinoids can induce at very low concentrations. This is of particular interest for non-target insects and for insect dependent species.

Keywords: Imidacloprid; Neonicotinoid; Lethal effect; Sublethal effect; Acute exposure; Chronic exposure; V-shape toxicity; LC50; Drosophila melanogaster, Flies; Bees; Mortality; Fecundity; Mating.

INTRODUCTION

Most of chemical pesticides are herbicides, fungicides or insecticides. There is increasing interest to reduce their impacts on the environment, especially on non-target species. Since the mid 90s, the class of neonicotinoids has become the most widely used and fastest growing family of insecticides worldwide.[1-4] This neonicotinoid family includes imidacloprid, thiamethoxam, clothianidin, thiacloprid, acetamiprid, dinotefuran and imidaclothiz.[5] Neonicotinoid insecticides interact with the nicotinic acetylcholine receptors (nAChRs) of the central nervous system. They target and bind to post-synaptic nAChRs of insects and to their body cell nAChRs.[4,6] They induce a neuronal hyper-excitation and accumulation of acetylcholine, leading to the insect's death within minutes.[1] Generally, lethal doses induce to tetanic contractions, with intense trembling (legs), body convulsions and paralysis. This is accompanied by nerve and muscle destruction.[7] They are significantly more selective towards insects than towards vertebrates.[8,9] As a general fact, flying insects were found to be the most vulnerable species to neonicotinoid pesticides.

Imidacloprid (IMI), [1-(6-chloronicotinyl)-2-nitroimino-imidazolidine], is one of the most representative chemical of the neonicotinoid insecticide family.[3] IMI has a very high activity against insects and lethal doses 50% (LD50) or lethal concentrations 50% (LC50) are very low, by topical and oral exposures. The chemical structure of IMI ensures its diffusion within treated plants, by xylemic and phloemic transport.[10] This systemic property gives IMI the advantage as a soil treatment and for treatment of seeds, with doses ranging from 50 to 100 g/ha, to protect the whole field.

The sublethal effects of IMI have already been studied in non-target insects[11]; mainly those of economic importance such as honey bees.[12-16] However, relationships between effects and mechanisms of action are complex and difficult to establish. In order to better understand the

effects of IMI, we used the drosophila model for which all parameters can be managed with confidence. The advantages of drosophila include a short life cycle as well as the efficiency and extensivity of tools about its genetics and genomics. Drosophila has already been used to investigate the genotoxic effects of pesticides such as IMI and acetochlor.[17]

Our main goal was to identify and to differentiate concentration ranges where sublethal and lethal effects occur. The LC50 was first determined from acute exposure for larvae and adults (females and males) of a fruit fly strain fully managed in our laboratory. Chronic exposure over 8 days defined chronic LC50 for females and males. By investigating lower concentrations, additional effects on the survival, mating and fecundity, were characterized.

EXPERIMENTAL SECTION

All experiments were conducted at 22°C ± 1°C. Test experiments (all tested concentrations of IMI) and controls were done in parallel (synchronicity). All experiments were performed over a 1.5 month period (March - April). All experiments were repeated at least six times. The number of flies tested for each data point is defined as N.

Strain and medium: A wild type stock of flies, named Orleans, derived from specimens caught in fields near Orléans (France) in 2000 and maintained in the laboratory by inbreeding, was used in this study. The stock of flies was maintained in our laboratory by mass culture at 22°C on a standard medium. The composition for 4L of standard medium was as follows: 362 g of cornmeal, 200 g of dry yeast (inactive and not hydrolyzed), 60 g of agar and 150 mL of a 10% solution of methyl-4-hydroxybenzoate (CAS number: 99-76-3) in ethanol. All

experiments were done with the same batch of cornmeal and yeast (Dominique Dutcher, Brumath, France).

Solvents, reagents and chemicals: All solvents (acetone CAS number: 67-64-1, acetonitrile CAS number: 75-05-8, dimethyl sulfoxide CAS number: 67-68-5, methanol CAS number: 67-56-, ethanol CAS number CAS number: 64-17-5 and water CAS number: 7732-18-5) were purchased from VWR (Fontenay-Sous-Bois, France) and are at least HPLC grade (ACS grade for DMSO). IMI (MW: 255.66 g/mol, Purity 99.5%) was obtained from CIL Cluzeau (Sainte-Foy-la-Grande, France). The starting solution (100 g/L) of IMI was prepared in DMSO as this solvent is a component of the commercial formulation[18] and because solubility of IMI in water is relatively low.[19] Other IMI solutions were obtained by dilution in distilled water. Test solutions of IMI were obtained by diluting this starting solution in water. DMSO was always present in test solutions and controls, at the same concentration, always lower than 1 % (v/v). Other reagents, such as salt compounds (KH_2PO_4), were analytical grade and obtained from Aldrich (Saint-Quentin Fallavier, France).

Acute toxicity: Males and females of three to four days old, in groups of about 20 flies, were transferred to vials without food for 6h, and then to vials containing a blotting paper moistened with a 5% sucrose (CAS number: 57-50-1) solution with the tested concentration of IMI. Flies were left in these conditions for 18h. The control groups were fed with 5% sucrose or 5% sucrose containing the same concentration of DMSO. For instance, DMSO was 1 % (v/v) for IMI = 3.91mM; DMSO was 10^{-3} % (v/v) for IMI = 3.91µM and DMSO was 10^{-6} % (v/v) for IMI = 3.91 nM. After 18h, all flies were transferred into vials containing a standard medium. After 8d, the flies still alive were counted. Series of ten concentrations,

ranging from 7.8 µM to 3.1 mM, were used for adults. Because we limited the DMSO content at less than 1 % (v/v), tests at higher concentrations were precluded.

To obtain the third-instar larvae, eggs were collected during a 3h period and kept at 22°C. Four days later, groups of 20 larvae were transferred into individual Petri dishes containing agar 2% and some yeast paste prepared with a solution of IMI at the tested concentration. The larvae were transferred 18h later into vials containing the standard medium. Larvae were kept on this medium and the surviving adults were counted. Control experiments were done with the same protocol but the yeast paste was prepared without IMI and with a solution where DMSO was at the same concentration as in the test solutions. Seven concentrations, ranging from 11.7 µM to 0.5 mM, were used for larvae.

Chronic toxicity: Males and females were tested separately. Groups of 20 flies, 3-to-4d old, were transferred into vials containing a fresh drosophila Instant Medium (DIM; Carolina Biological Supply, Burlington NC) prepared with distilled water containing the test IMI concentration (1.5 g rehydrated with 4.5 mL of test solution). Flies were kept in these vials continually. Flies, still alive 8d later, were scored.

For larvae, the eggs were collected during a 3h period. Immediately after hatching, larvae were transferred into vials containing fresh DIM rehydrated with the test solutions. Larvae were maintained on this medium, and the adults which emerged were counted. Control experiments for adults and larvae were done by using solutions at the same concentration of DMSO as the test solutions.

Mating tests: Sets of 5 virgin males and sets of 5 virgin females (all flies < 6h old) were randomly recovered from the stock in presence of IMI. They were placed into vials containing the fresh DIM with IMI. After 5 days, five females and five males were transferred without

anaesthesia into empty vials. These vials were observed for 20 min to determine how many females, in each vial, had mated. Note that this is the total duration of mating for drosophila. Because males and females used in this test were exposed to IMI during their rearing, we chose concentrations which allowed their larval development, far below the chronic LC50. Control experiments, using DMSO at the same concentration, were run in parallel. For consistency, N represents the number of females for mating tests.

Fecundity tests: virgin males and virgin females (< 6h old) were randomly selected from the stock in presence of IMI and transferred in vials containing the fresh DIM with IMI. Males were raised individually and females were raised in groups of 5 flies per vial. After 5 days, one female and one male were transferred into a vial containing the standard medium. The vials were observed until the pair copulated, after which the male was removed. Each female could lay eggs for 24h in this vial. Then the female was transferred into a new vial for 24h where it could lay further eggs. This transfer was repeated five times. At the end, the female was transferred into a new vial where it could lay eggs until day 15. Adult emergence was scored for all vials. We only used data from females that produced offspring. We checked that the number of females producing offspring were as numerous in test as in controls. As for the mating tests, we chose low concentrations. Control experiments, using DMSO at the same concentration, were run in parallel.

Analytical measurements: Adult flies (4-to-5d old) were starved for 6h and were placed in a vial containing blotting paper moistened with the IMI test solutions. Immediately after the knock-out effect, flies were frozen at -80°C by batches of 20. Batches of flies (directly taken from the freezer) were ground in a glass test-tube containing 1 mL acetonitrile with a Turrax 5G (IKA) for 2 min (twice). After evaporation of the solvents, the residue was transferred

with acetone in order to proceed to a further purification step by SPE on Bond Elut 500 mg/3 mL, purchased from Varian Inc and conditioned with acetone (2 mL). The first five fractions were collected (5x5 mL), evaporated and solubilised in 200 µL methanol. 50 µL of this solution was then injected in a HPLC column through a rheodyne type valve. HPLC/UV analyses were performed, according to Obana *et al.*,[20] with a Merck apparatus (L-6200A Intelligent Pump; L-4000 UV Detector; D-2500 Chromato Integrator) coupled with a C18 HPLC column, 3 µm diameter (250 x 4.6 mm i.d.) purchased from VWR. IMI was detected at 270 nm with a retention time of 18.4 min. The calibration curve was calculated from 5 points (1, 10, 100, 400 and 800 mg/L), with $R^2 = 0.9988$.

Statistical analyses: Data were statistically analyzed with the R software from R Core team (2013), R Foundation for statistical Computing, Vienna, Austria (http://www.R-project.org/). A general linear model (GLM) was used with a logistic link function (logit). The model has investigated main effects of i) the IMI concentration, ii) the sex of flies and iii) concentration-sex interactions, for survival data after chronic exposure. The model has investigated only the effect of IMI concentration for mating data. Additionally, comparison tests of independent proportions were used to identify significant differences between each experimental and control groups. For fecundity data, Mann-Whitney tests were used for comparisons. All these comparison tests were considered bilaterally, i.e. considering the possibility of positive or negative effects. The statistical significance for all comparisons was set at $p < 0.05$ (*), $p < 0.01$ (**) and at $p < 0.001$ (***).

We also used Stat Graphics XV (15.2.14) from SIGMA PLUS (Levallois-Perret, France) to estimate the LC50 values and their 95% confidence interval (CI95). This was done by using the probit method developed by Finney.[21]

RESULTS

Acute exposure

Survival: Insecticidal effects of IMI on larvae and adult drosophila were tested after acute treatment (18h). In the control experiment (DMSO control), the average survival rate was 95% for adults and 82.5% for larvae. For adult females, a LC50 value was not determined, because only 31% of them died at the highest concentration used (3.1 mM). This indicates the high resistance of the drosophila strain used in this study. In contrast, at the same concentration (3.1 mM), 91% of adult males were killed, allowing us to determine the corresponding LC50 value at 1304 ± 92 µM (Table 1).

For larvae, we did not discriminate between males and females. The number and gender of adults emerging were scored. No bias concerning female versus male was observed in the offspring, suggesting that the two sexes were equally killed by IMI at this larval stage. It should be noted that mortality induced by IMI occurred during the larval stages, as no lethality was observed in pupae stages. When compared to LC50 for adult males (1.3 ± 0.1 mM), the LC50 for larvae is 8 times lower (157 ± 25 µM), suggesting a higher acute toxicity of IMI for larvae.

Analyses of flies: To estimate the residual amounts of IMI in insects, we performed measurements after intoxication of adults (of both sexes) at two IMI concentration levels (Table 2). Results were normalized with respect to mass ratio between male and female (1:1.4). We found the same amount of IMI in adult males and females, 452 ± 142 ng/male and 475 ± 111 ng/female, respectively, this when feeding was done on solutions at 3.1 mM (800 mg/L). Values were 184 ± 24 ng/male and 163 ± 36 ng/female when feeding was done on

solutions at 1.3 mM (333 mg/L). Thus, masses of IMI in males and in females were statistically equivalent and were proportional to the exposure levels.

Chronic exposure

Survival: We first determined LC50 values. The control experiments (with DMSO) always displayed a survival rate over 94% for adults and it was over 83% for larvae. For the highest concentrations, results revealed typical sigmoid curves for which mortality increased sharply with concentrations (Figure 1). Table 1 shows LC50 values for adults and larvae. A distinction between sexes was made for adults. For adult males, the LC50 is 45 ± 5 µM instead of 18 ± 1.5 µM for adult females. These data indicate that females seem slightly more sensitive than males, after chronic exposure to IMI. The chronic LC50 was determined at 3 ± 0.3 µM for larvae (Table 1). IMI is then more toxic (from 6 to 15 times) for larvae than for adults, after chronic exposure.

For adults we observed a particular shape of the survival curve, this shape seemed to be conserved between males and females but shifted with respect to the concentration scale (Figure 1). Statistical analysis (GLM) confirmed that there are significant effects of i) the IMI concentration and ii) the sex of flies and iii) the concentration-sex interactions. The shape (in form of V), showed a highly significant increase in mortality for females at 3.91 nM and 39.1 nM, and for males at 39.1 nM ($p < 0.001$). At these concentrations, the maximum value of mortality was 28% and 27% for females and males, respectively.

Mating: We studied the mating rate (during 20 min) of sets of 5 couples after chronic exposure of flies during their whole life (larvae and adult). This was done between 0.0196 nM and 391 nM of IMI (Figure 2). Data suggested that IMI could induce an increase of the mating rate at 0.391 nM. At this concentration, the mean number of females which had mated

within 20 minutes was 4.1, instead of 3.1 in the control experiment. However, statistical analysis (GLM) of all data points did not indicate any effect of IMI within this large concentration range. But, when comparing each data point with respect to the control, significant differences (30%) were confirmed at 0.391 nM ($p < 0.001$) and at 1.96 nM ($p < 0.01$).

Fecundity: We counted the number of offspring per female after chronic exposure of flies during their whole life (larvae and adult). This was done for various concentrations of IMI: from 0.391 nM to 391 nM. A first set of experiments included both males and females exposed to IMI (Figure 3A). Here we observed a significant decrease in fecundity at 1.96 nM, 3.91nM and 39.1 nM ($p<0.05$) when compared to controls. At a lower concentration (0.391 nM) or at a higher concentration (39.1 nM or 391 nM), there was no significant statistical difference between controls and exposed flies. To assess the origin of this decrease in fecundity (effects on males or on females), we also exposed to IMI (3.91 nM) only males, or only females. The result showed that the decrease in fecundity can be attributed to the exposure of female flies only ($p < 0.01$), whereas there is no difference with control when only males were exposed (Figure 3B).

In order to better understand this decrease in fecundity, we compared the rate of hatching of embryos laid by control females, to that of exposed females. No significant difference was observed and about 95% of embryos had hatched into larvae in both cases. We also checked the possible lethality during larval or pupal stages. As matter of fact, no significant lethality was observed during these two developmental stages (data not shown).

DISCUSSION

Effects of IMI and LC50 (acute and chronic)

We observed that the LC50 after chronic exposure was 29 times lower for adults and 43 times lower for larvae, when compared to an acute treatment (Table 1). In a previous paper which studied the genotoxic effects of IMI and of acetochlor in *Drosophila melanogaster*, the LC50 for IMI was determined after acute and chronic treatment on a mutant strain kept in laboratory.[17] When comparing adults, the Orléans strain is more resistant to IMI than the mutant one. Here, the acute LC50 (adults) is 10 times higher than the value from Frantzios *et al.* In contrast, the chronic LC50 for adults of the Orléans strain is slightly lower than the chronic LC50 determined by the same authors (Orléans: 17.6 µM for ♀ and 44.9 µM for ♂ versus 60 µM for the mutant strain). Note that Frantzios *et al.* did not distinguish between males and females. Concerning larvae, the Orléans strain also appears more resistant after acute treatment (LC50 157 ± 25 µM versus 75.5 µM), but less resistant after chronic treatment (LC50 3 µM versus 26.7 µM). The discrepancies observed between the two studies are probably explained by differences in the genetic background of each strain of flies. However, the Orléans strain appeared more resistant to mortality than the Oregon-R strain (data not shown) and as resistant as the Hikone-R strain, the latter one known to be resistant to DDT and IMI.[22] It cannot be excluded that the Orléans strain could be issued from wild type flies selected for resistance from 1994 (introduction of IMI on the local market) to 2000. Note that our study was performed in spring. It would be interesting to compare results obtained in various seasons and for various drosophila strains.

The analysis of the survival curves after chronic exposure revealed that, above 3.91 µM, mortality was directly related to the logarithm of concentrations (Figure 1). In this case, data have typical representations with sigmoid shapes and LC50 values were determined as

mentioned above. However, it can be observed that more than one fourth of flies died at 3.91 nM (females) and 39.1 nM (males). Three hypotheses can be mentioned related to these results. First, the processes of detoxification of IMI (for instance by cytochrome P450) would not be initiated so efficiently (concentration threshold) so a much larger fraction of the consumed IMI could reach the nAChRs. Second, IMI could bind to different receptors with different affinities (low and high affinity). Third, it cannot be excluded that a synergistic effect between DMSO and IMI could have occurred, but is unlikely because such a synergy i) has little chance to only occur for very low amount of DMSO and ii) has little chance to differ between males and females. However, data are still lacking to validate these hypotheses.

Differences of LC50 depending on fly sex

In our experiments, females appeared more resistant than males after acute treatment. We tested the hypothesis that females could take less IMI than males. For this, we have determined the mass of IMI in the body of females and males. Therefore, we showed that the two sexes contained the same mass of IMI per insect (Table 2). Thus, we can make the reasonable assumption that both sexes have taken about the same quantity of IMI, although differences (amount, frequency) in food intake between the two sexes cannot be excluded. It is interesting to note that, when exposure was chronic, females are less resistant than males. Such a difference between sexes was also observed for various xenobiotics, as for example, caffeine,[23] cycloheximide,[24] endosulfan and malathion[25] or cypermethrin and fenvalerate.[26]

Differences of LC50 between larvae and adults

In larvae the LC50 was about tenfold lower than the corresponding ones for adults, for both modes of intoxication, demonstrating a higher sensitivity of larvae to IMI (Table 1). An explanation is that larvae are in continuous contact with IMI during the experiment.

Therefore, IMI could also diffuse through the integument and the digestive tract, leading to both topical and oral exposure. In contrast, IMI enters mainly through the digestive tract of adults. According to this hypothesis, a higher amount of IMI should be found in the larval body than in the adult body. Analyses were done, and we quantified IMI after acute exposure of larvae. The results suggested that the amounts of IMI were similar in larvae and adults. However, if results are normalized according to the body weight, for an identical feeding concentration, larvae were submitted to higher doses of IMI than the adults. Such a difference of exposure could account for the difference of LC50 between larvae and adults.

Sublethal effects

Chronic exposure at very low concentrations of IMI showed significant effects on mating, with a maximum of + 30% at 0.391 nM (Figure 2). It has been shown that the drosophila courtship is a behaviour affected by experience during the first days of adult life. Moreover, nAChRs are exclusively neuronal in drosophila.[27,28] Therefore, we can expect that exposure to very low doses, which affects neuronal plasticity during the early life, can result in alteration of the mating behaviour. A similar effect has already been observed in drosophila after exposure to lead.[29]

Significant effects were also revealed when studying the fecundity after chronic treatment of both genders with IMI (Figure 3A). This decrease of fecundity (maximum 16%) also displayed a shape in form of V and was linked to exposure of females only (Figure 3B). Several hypotheses can be proposed to explain this result. Firstly, chronic exposure to IMI could affect oogenesis, as it is the case for cocaine.[30] However, a first inspection of ovaries has not revealed evident anomalies of egg chambers. Secondly, exposure to IMI could indirectly induce some paralysis of the muscle fibers of the reproductive tract. However,

Middleton *et al.* have demonstrated that the contraction in the drosophila ovary is under octopaminergic neuromodulation.[31] Thirdly, the continuous presence of IMI in the medium could alter the hormonal status of females and could affect egg production. For instance an increase of ecdysone reduces egg production.[32]

Finally, IMI induced sublethal effects and mortalities on this drosophila strain far below the LC50. This was substantiated when the exposure mode for larvae, or adults, was chronic. The Lowest Observed Effect Concentration (LOEC) was 0.391 nM and concerned mating. LOEC was 4 orders of magnitude lower than the chronic LC50 for females. It was 5 orders of magnitude lower than the chronic LC50 for males. Such effects of IMI are certainly not restricted to our drosophila strain. For instance, there is also 5 orders of magnitude between the acute LC50 and significant mortalities after chronic exposure of bees over 10d.[33] Such effects are also consistent with the reduction of colony growth and the drastic reduction of queen production for bumble bees exposed to field-realistic concentrations of IMI.[34]

Implications and perspectives

Drosophila melanogaster may be a convenient model for toxicity studies of chemicals such as IMI. It is convenient for determining chronic LC50 which is a relevant parameter for realistic exposure of non target species. It also allows time-to-effect studies which have been exemplified by Tennekes and Sanchez-Bayo for neonicotinoids[35] in the cases of aquatic invertebrates and other arthropods. These latter studies are of particular importance because IMI can have direct effects on pollinators and birds[36] or indirect effects on insectivorous species.[37, 38] In this view, two recent studies focused on adverse effects of neonicotinoids on large ecosystems including pollinators, aquatic species and mammals.[39, 40] New works for

studying other nicotinoids and other systemic insecticides should be performed by using drosophila as a laboratory model.

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Note

The authors declare no conflict of interest.

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Table 1. Lethal concentrations 50% (LC50 in µM) of imidacloprid for *Drosophila melanogaster* (Orléans wild strain).

	LC_{50} (µM)[a]		
Mode of exposure	Adult males	Adult females	Larvae
Acute	1304 ± 92	> 3100*	157 ± 25
Chronic	45 ± 5	18 ± 1.5	3.0 ± 0.3

[a]LC50 were calculated from sigmoid mortality curves. Mortalities were counted after 8d following an acute exposure (18h) or chronic exposure (8d). The LC50 for adult flies (males and females) and for larvae were obtained with the same experimental conditions. The LC50 and their corresponding 95% confidence intervals (CI95) were determined by probit analysis (see the experimental section).

*Estimated value because of the limited solubility of imidacloprid with respect to the experimental protocol.

Table 2. Amounts of imidacloprid (in ng) per adult drosophila, measured by chemical analysis.

	Imidacloprid content (ng) per adult drosophila*[a]	
Feeding concentration (mg/L)	Males	Females
800	452 ± 142	475 ± 111
333	184 ± 24	163 ± 36

*Chemical analyses were done following an acute exposure and after the knock-out effect.

[a]Confidence intervals at 95% (CI95), issued from statistical analysis, are reported.

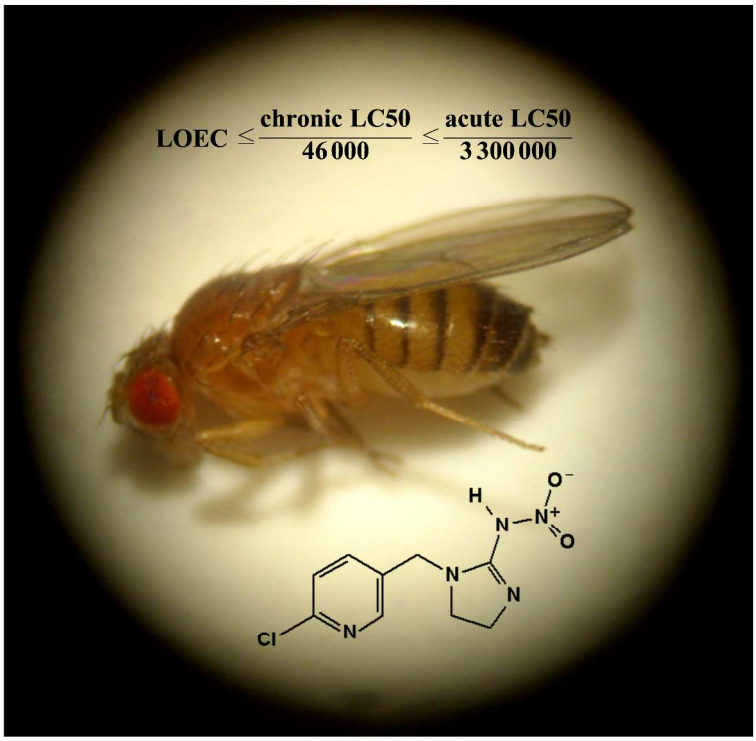
Figure Captions

Figure 1. Average ratios of surviving drosophila after chronic exposure. Data are reported for adult flies: males (■) and females (▲). Concentrations of imidacloprid ranged from 0.391 nM to 0.391 mM. Ratios are given from the number of flies still alive, after chronic exposure to imidacloprid (8d), over the number of flies exposed (see the experimental section). N: number of flies. Bars corresponding to 95% confidence intervals (CI95) are reported for each data point. Ratios for controls are indicated on the vertical axis: males (□) and females (Δ). Significant differences are indicated in the low concentration range only (*** when $p < 0.001$).

Figure 2. Average percentage of mating, depending on imidacloprid concentration. Mating was counted during a period of 20 min, after chronic exposure to imidacloprid (see the experimental section). Concentrations of imidacloprid were between 0.196 nM and 391 nM. For clarity, the horizontal axis has a non linear scale. Result for controls are given on the left (controls: white; tests: grey). N: number of females tested. Bars corresponding to 95% confidence intervals (CI95) are reported for each data point. Significant differences are indicated (*** when $p < 0.001$ and ** when $p < 0.01$).

Figure 3. Average number of offsprings per female. Offsprings were counted after chronic exposure to imidacloprid (see the experimental section). Concentrations of imidacloprid were between 0.391 nM and 391 nM. N: number of females tested. Bars corresponding to 95% confidence intervals (CI95) are reported for each data point. Significant differences are

indicated (** when $p < 0.01$ and * when $p < 0.05$). In (A), both male and female flies were exposed (controls: white; tests: grey). In (B), only one gender was exposed at a concentration of 3.91 nM (controls: white; tests: grey; ♂: males; ♀: females).



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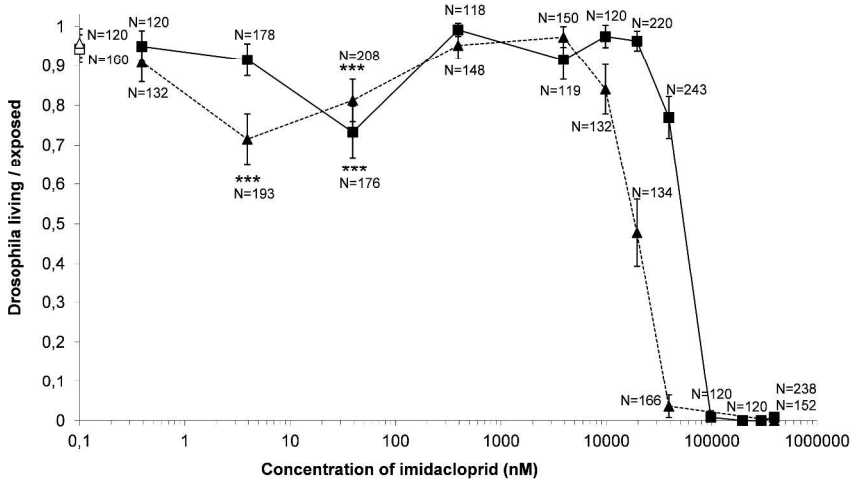

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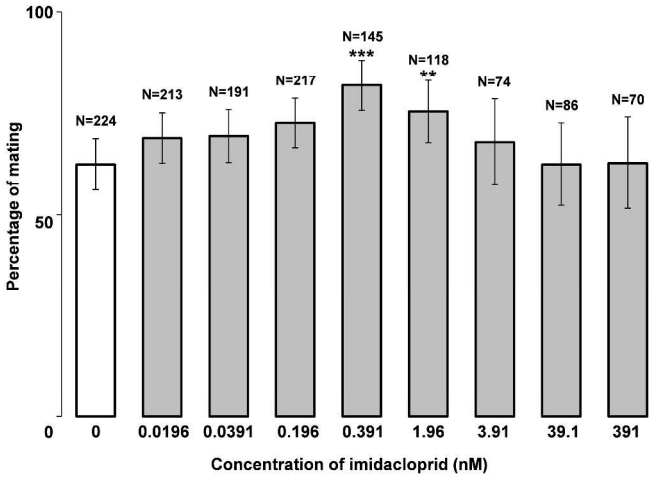

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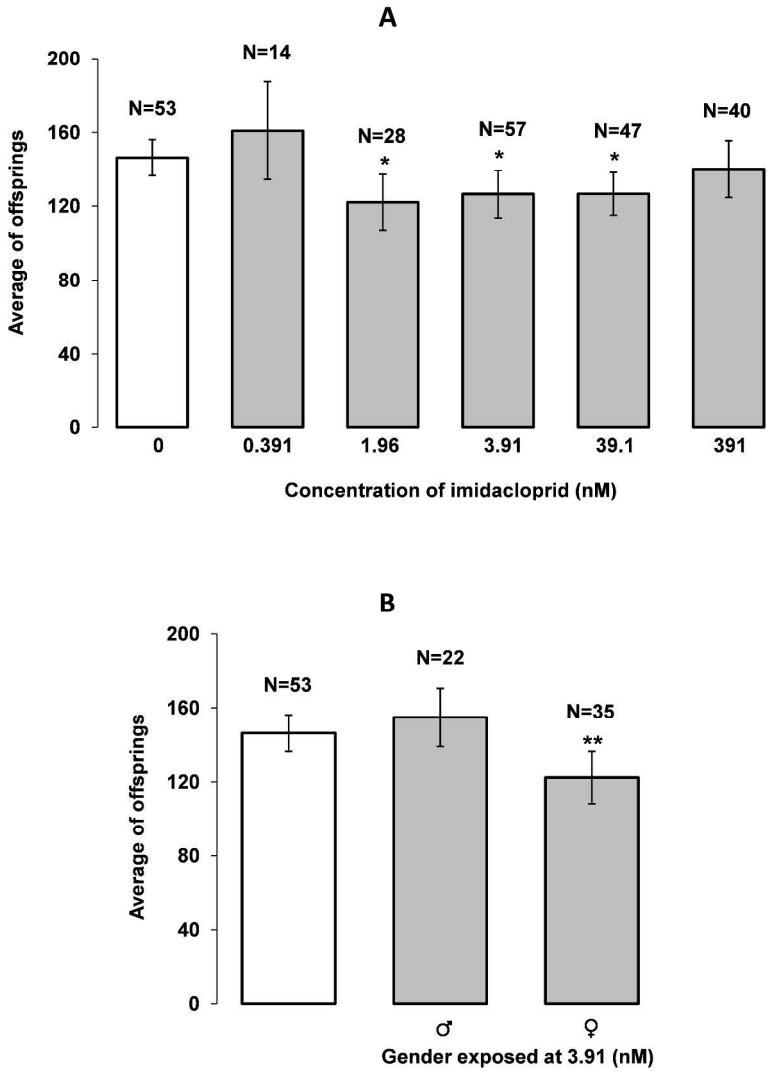

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