



OPEN Intracellular calcium handling dysfunction contributes to behavioural deficits leading to mortality of honey bees after acute contact exposure to the insecticide cyantraniliprole

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Synthetic insecticides are recognized as a major factor contributing to the global decline in insect abundance and diversity. Within this context, anthranilic diamides have gained increasing market share in Europe since 2018. We investigated the second-generation diamide cyantraniliprole, which induces aberrant cytoplasmic calcium release in honeybee skeletal muscle cells. In vivo assays demonstrated that acute contact toxicity varied with exposure site, being more severe when applied to the abdomen, antennae, or ventral thorax compared with the dorsal thorax. Following thoracic exposure to sublethal doses, continuous 21-h monitoring revealed a dose-dependent alteration of behavior, with a reduction in locomotor activity, including maximal velocity and total distance traveled. Notably, at the lowest concentration, antennal exposure elicited stronger behavioral impairments, indicating potential disruption of sensory processing and environmental cue perception. Importantly, cyantraniliprole and its parent compound, chlorantraniliprole, also triggered anarchic calcium mobilization in mammalian skeletal muscle fibers, highlighting potential cross-taxa effects. These results strengthen previous evidence of diamides toxicity in bee cardiomyocytes and neurons and emphasize significant gaps in current risk assessment frameworks. Collectively, our findings indicate that anthranilic diamides represent not only an ecological threat to pollinators but also raise concerns for mammalian health, warranting more stringent evaluation of this insecticide class.

Keywords Honey bee, Insecticide, Anthranilic diamide, Cyantraniliprole, Intracellular calcium channels, Ryanodine receptors

Insect decline has recently been recognized as a serious threat to global biodiversity and food production¹. One of the main causes of this decline is the overuse of neurotoxic insecticides against phytophagous insects in agricultural landscapes². The most widely used neurotoxic insecticides target ion channels^{3,4}. These ion channels are large proteins that form pathways for ion fluxes (primarily Na⁺, K⁺, Ca²⁺, and Cl⁻) through cell membranes and influence electrical excitability in important tissues, such as the peripheral and central nervous systems, the cardiac and muscular systems, or glands^{5,6}. Insecticides that interfere with ion channels, therefore disrupt vital functions and act quickly on insects. Calcium channels are the most recent target for synthetic insecticides, with new molecules being developed by companies under the name of ‘diamides’⁷. Agrochemical companies promote diamides as a credible alternative to older insecticide classes due to their unique molecular mode of action, translaminar mobility into leaves, and systemic activity⁸. They are transported through plant

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fluids, extending their toxic effects on insects⁹. By 2021, diamide sales accounted for 13% of the global market share in dollars⁴. Sales of anthranilic diamides increased significantly in Europe between 2017 and 2021, rising from 13 to 63 tons¹⁰. Recently, a combination of in vitro and in vivo approaches has helped to characterize their mode of action and the harmful effects of low doses on the behaviour of honey bees, a model insect in neurophysiology and toxicology. In honey bee central neurons, peripheral neurons, skeletal muscle cells, or cardiomyocytes exposed to chlorantraniliprole (a first-generation anthranilic diamide) or flubendiamide (a phthalic acid diamide), chaotic intracellular calcium mobilization was observed^{11–13}. These molecules target intracellular calcium release channels, known as ryanodine receptors (RyRs)^{8,14}. Recent experiments suggest that voltage-gated calcium channels (VGCCs), located in the plasma membrane of neurons, cardiomyocytes, and skeletal muscles, may also serve as secondary targets, especially at higher concentrations^{11,13}. Both VGCCs and RyRs are essential for excitation–contraction coupling in honey bee muscles and the heart^{11,13,15}. They are also involved in central and peripheral neuronal functions^{12,16–18} and in compound eyes¹⁹. Honey bees possess four genes encoding the pore-forming units of VGCCs, while a single *ryr* gene is expressed in both honey bees and wild bee species^{20–23}. In contrast, vertebrates have three *ryr* genes that share only 45% homology with insect *ryr* genes²⁴. Early studies described diamides as being (*sic*) ‘specific to insect ryanodine receptors and [not affecting] mammalian skeletal muscle ryanodine receptors’⁷. Further research confirmed (*sic*) that ‘the phthalic diamide flubendiamide and anthranilic diamides chlorantraniliprole and cyantraniliprole act at an allosteric site of the RyR to activate calcium release in insects but not mammals’²⁵. However, this clear-cut view of molecular specificity has been revised, showing that diamides can also act on mammalian RyR channels, with selectivity observed in heterologous expression systems. Evidence also indicates that diamides interfere with rabbit RyRs, as demonstrated through electrophysiological studies on purified RyRs using the lipid bilayer technique^{26,27} or in isolated sarcoplasmic reticulum vesicles²⁸. These findings were observed in wild-type RyRs, but several human diseases are associated with mutated calcium release channels. These diseases, called ryanodinopathies, include Malignant Hyperthermia (MH), Central Core Disease (CCD), Calcium Release Deficiency Syndrome (CRDS), among others²⁹. There exists a possibility that ingested diamides, accumulated in plants or meats, can differentially affect these naturally-mutated ion channels, whilst intoxications with a large dose of diamide have been reported in humans³⁰. Overall, not only diamides could thus constitute a general threat for vertebrates and invertebrates, but their effects in organisms carrying a RyR mutation should be even more scrutinized in order to better take into account any increased susceptibility leading to an underestimated toxicological danger in populations at risk.

The first-generation diamide Chlorantraniliprole was more toxic to bees when either the dorsal abdomen or antenna was exposed as compared to the dorsal thorax¹¹, the latter being the classical exposure zone in the mandatory contact toxicity test³¹. In the context of insect decline, to better predict the harmful effects of marketed insecticides on bees, the present study aimed to more precisely document this differential sensitivity with the second-generation diamide, cyantraniliprole. Following the European Food Safety Authority (EFSA) recommendation, pointing out gaps in risk assessment, we monitored effects both at high doses causing mortality and low doses that could alter honey bee behaviour. Since cyantraniliprole’s action on insect RyRs has so far only been described on heterologously expressed RyRs, the second objective of the study was to explore the sensitivity of honey bee muscle RyRs in situ. Finally, we also measured the in situ sensitivity of mammalian RyRs to cyantraniliprole in muscle cells from either normal mice or from a pathological mouse model carrying a MH-type mutation in the RyR receptor (Y522S). Part of this work was presented at the Journal of General Physiology E–C Coupling Meeting³².

Results and discussion

Acute contact toxicity of cyantraniliprole to honey bees depends on the exposure route

Our earlier study suggested that the first-generation anthranilic diamide chlorantraniliprole was more toxic when applied onto specific zones of the bee body¹¹. To explore in more detail, the toxic potency of cyantraniliprole on vital organs of the bee, such as the heart, the antennae, flight muscles, and thoracic ganglia, we designed an experiment in which bees were exposed through body areas close to these organs. Exposure zones were as follow (Fig. 1): dorsal abdomen (the heart tube lies right beneath the dorsal abdominal cuticle), the left antenna (an important organ in olfaction and gustation, as well as other types of chemical or physical stimuli), dorsal thorax (flight muscles are concentrated beneath the dorsal cuticle), ventral thorax (thoracic motor ganglia are localized right beneath the cuticle of the ventral thorax compartment). After exposure, mortality was assessed in individual arenas to avoid cross-intoxication that may arise when bees rub against each other in collective cages. Figure 1A shows four bees, each one enclosed in an individual arena (closed Petri dish, 5 cm in diameter). Each arena contained a feeder, and no mortality was observed in any of the control bees at 24 h after exposure, regardless of the exposure zone (Fig. 1B, $n = 15$ control bees for each of the 4 exposure zones). When dorsal abdomen, ventral thorax or antenna were exposed to cyantraniliprole ($n = 18$ bees for each dose and each zone), a strong mortality was observed at 100 ng/bee ($p = 0.0361$, $p < 0.0001$, $p = 0.0032$, respectively) and 250 ng/bee ($p < 0.0001$ for all locations, exact Fisher tests). Cyantraniliprole appeared clearly less toxic when applied on the dorsal thorax, since no mortality was observed 24 h after exposure at any tested dose (up to 250 ng/bee, exact Fisher test $p > 0.999$). The dose 25 ng/bee did not induce significant mortality for any exposure zone ($p = 0.0748$ for antenna and $p > 0.999$ for all other locations, exact Fisher tests).

Estimated cyantraniliprole LD₅₀ (obtained with a logistic regression, see Methods) were 80, 98 and 148 ng/bee when bees were exposed via ventral thorax, antenna, and dorsal abdomen, respectively. A delayed toxicity was observed after dorsal thorax exposure to 250 ng/bee with 28% dead bees at 72 h at this dose, while no bees died in the control group ($p = 0.0361$, exact Fisher test; Supp Fig. 1). Mortality increased slightly for the other exposure routes as well (up to 83, 94, and 94% at 72 h after exposure of dorsal abdomen, antenna, and ventral thorax to a dose 250 ng/bee).

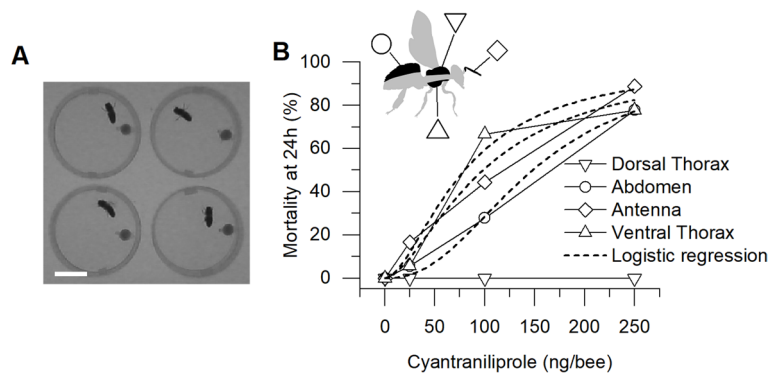


Fig. 1. Contact acute toxicity of cyantraniliprole to honey bees depends on the exposure route. **(A)** Bees survival assessed in separate chambers to avoid interindividual aggressiveness and/or unwanted overexposure via cuticle-cuticle friction. Four bees (dark shape) enclosed in their individual arena (scale bar: 2 cm). A feeder provided food to each bee (round grey shape at 3 o'clock in each arena). **(B)** Higher toxicity of the insecticide 24 h after exposure via antenna, dorsal abdomen or ventral thorax, as compared with dorsal thorax. Logistic regressions (dashed lines) gave LD_{50} in the range 80–148 ng for these 3 locations.

The lower toxicity of cyantraniliprole through dorsal thorax exposure prompted us to verify the toxicity of cyantraniliprole in conditions mimicking to the classical OECD test 214³¹. An additional experiment was thus performed with bees that were kept in collective cages after acute contact exposure through the dorsal thorax (10 per cage, 3 replicates). In this experiment, significant mortality was observed, starting from dose 250 ng/bee (43%, Fisher exact test, $p = 0.0004$), and LD_{50} at 72 h was 885 ng. Ninety percent of bees were killed by a dose of 6000 ng/bee. These results confirm that cyantraniliprole is also toxic when applied onto the dorsal thorax.

Alteration of locomotor performances after acute exposure of bees to cyantraniliprole

Earlier studies have demonstrated that the deleterious effects of anthranilic diamides not only result from lethal effects at high doses, but also from long-lasting behavioural impairments after exposure to a low dose^{33,34}. These symptoms can last for several days. Behavioural deficit can also reappear as a relapse, several days after a single exposure¹¹. Here, twenty-four hours after exposure to cyantraniliprole via dorsal thorax (0–250 ng/bee), all bees kept in individual arenas were still alive (Fig. 1B), however, their behaviour was clearly affected. The set-up shown in Fig. 1A allowed for an objective assessment of the level of behavioural alteration for each bee during 21 h after exposure (constant dark cycle, see Methods). Control bees (which received only the vehicle, *i.e.* acetone), had phases of activity interrupted by phase of inactivity (the threshold for inactivity was set at speed 3 mm/s, see Methods). Actograms from two representative control bees are shown in Fig. 2A and B. No circadian rhythm (day/night activity pattern) was observed in these young bees, as expected from earlier studies³⁵. Both bees showed an increase in speed over time, representative of the tendency observed in the sampled bees (Fig. 2C shows an increase in the median speed calculated from 15 control bees). The percentage of active bees increased from ~20% to ~50% over the period of observation, which is another indication of the progressive acquisition of locomotor skills over time in young bees (Fig. 2D). Three proxies of bees' activity were extracted from individual 21h-actograms: (i) maximal speed (V_{max}), (ii) percentage of time spent in activity, (iii) total distance covered. On average, the maximal speed of control bees was $24.9 \pm 1.5 \text{ mm}\cdot\text{s}^{-1}$ and the percentage of time spent in activity was $44 \pm 4\%$ (Fig. 3A and B, white dots, $n = 15$ bees). For control bees, the total distance covered was $505 \pm 62 \text{ m}$ (Fig. 3C). After exposure to cyantraniliprole, performances were significantly depressed at doses 100 and 250 ng/bee. On average, maximal speed was decreased significantly to $18.3 \pm 1.1 \text{ mm}\cdot\text{s}^{-1}$ and $14.5 \pm 1.5 \text{ mm}\cdot\text{s}^{-1}$ at 100 and 250 ng/bee, respectively (Anova and Tukey test, $p = 0.0149$ and $p < 0.0001$, respectively, see Fig. 3 legend for all statistics). A neat decrease in V_{max} was observed at these two doses (–27 and –42%, respectively). At 25 ng/bee, the same tendency was observed, but the difference was not statistically significant ($p = 0.6766$). Total distance covered was altered by cyantraniliprole as well, with a significant decrease to 263 ± 25 and $204 \pm 28 \text{ m}$ after exposure to 100 and 250 ng/bee, respectively (Kruskal–Wallis and Dunn's test, $p = 0.0342$ and $p < 0.0001$). Thus, a decrease in the total distance by 48 and 60% was observed at these two doses, respectively. Again, at 25 ng/bee, the same decrease tendency was seen, but the difference was not significant ($p > 0.9999$). Neither the percentage of activity nor the amount of food ingested were significantly different between control bees and bees exposed to cyantraniliprole (Fig. 3B and D, Anova and Kruskal–Wallis tests, respectively, see Fig. 3 legend for statistics). A Supplementary Fig. 2 illustrates actograms from bees exposed to doses shown in Fig. 3.

Results shown in Fig. 1 identified a higher lethal toxicity of cyantraniliprole as a function of specific zones of the bee body. The European Food Safety Authority (EFSA) pointed out shortcomings in the evaluation of non-lethal effects of chlorantraniliprole in honeybees³³. In order to further address EFSA concerns, the next set of experiments describes behavioural effects after exposure through 4 different zones to 25 ng/bee, a dose causing no significant mortality during 21 h. Data presented in Fig. 3A at dose 25 ng/bee (exposure: dorsal thorax) were compared with data obtained with bees exposed to three other zones: ventral thorax, dorsal abdomen, antenna (Fig. 3B, C and D, respectively). Fifteen control bees and eighteen bees exposed to 25 ng/bee were observed for each exposure zone. For each zone, the mean cumulated distance covered was normalized to the value measured

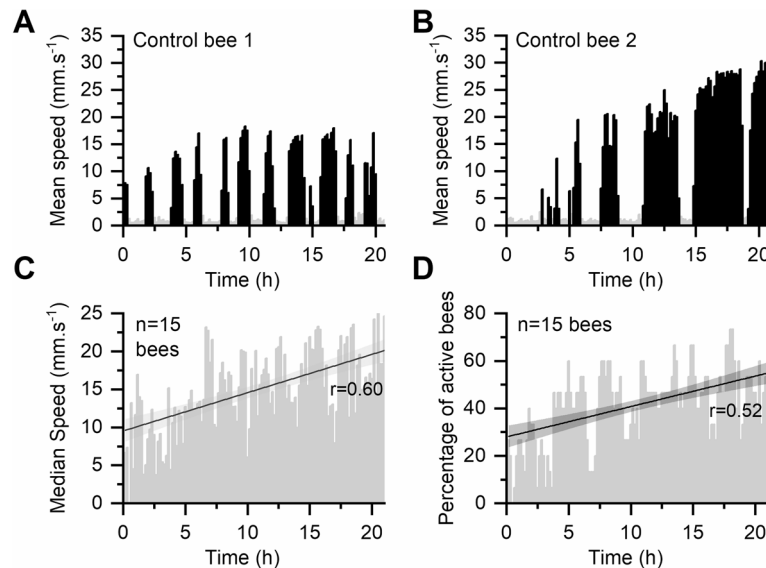


Fig. 2. Monitoring of control bees' behaviour and locomotor performances. (A and B) representative actograms from two control bees (1 μ l acetone applied on the dorsal thorax) over the 21 h observation period in dark conditions. Observation started at 10 a.m. The mean speed reflects the level of activity every 10-min bin (dark grey bars). Light grey bars show periods of inactivity (mean speed < 3 mm/s). Speed increased over time (8 to 18 mm/s for Bee #1 and 7 to 30 mm/s for Bee #2). (C) The speed of control bees consistently increased (median speed calculated from $n = 15$ bees). Linear regression, 95% confidence interval and Pearson's r coefficient ($p = 3.02 \cdot 10^{-13}$) indicate a positive correlation between time and improvement of locomotor skills. (D) The percentage of active control bees globally increased over time as well ($p = 6.15 \cdot 10^{-10}$).

at 21 h in control (Fig. 4, $n = 18$ bees). At 25 ng/bee, behaviour was altered only for exposure through the dorsal abdomen and the antenna (Mann–Whitney test, $p = 0.033$ and $p = 0.0001$, respectively). The antenna was found to be the most sensitive, with a decrease of 51% of the total distance covered, which is in line with earlier results showing sensitivity of antennal neurons to diamides¹².

Cytoplasmic calcium increase in bee skeletal muscle cells exposed to cyantraniliprole

Earlier results showed that anthranilic diamides exert their toxic mode of action on several bee cell types in vitro, such as peripheral and central neurons, cardiomyocytes, and skeletal muscle cells^{11–13}. In order to explore cyantraniliprole modes of action, single muscle cells loaded with the calcium indicator Fluo-3 attached to the bottom of the Petri dish were perfused with extracellular solutions (Fig. 5A). In response to perfusion with increasing cyantraniliprole concentrations (10 s exposure), fluorescence increases as a result of cytoplasmic calcium variations (Fig. 5B). Intracellular Ca^{2+} increased gradually with the concentration of cyantraniliprole (Fig. 5C). At the highest concentration tested (3300 nM), 21% cells contracted and in this specific case, fluorescence variation was measured before contraction (Fig. 5D).

Cytoplasmic calcium increase in mouse skeletal muscle fibers exposed to cyantraniliprole and chlorantraniliprole

Since cyantraniliprole strongly affects calcium homeostasis in bee muscle cells isolated from the legs, we performed an additional set of experiments with diamide insecticides on adult mouse skeletal muscle fibers, in order to compare effects of these molecules on calcium homeostasis in insects and mammals. Muscle fibers were isolated from the *flexor digitorum brevis* muscle (*fdb*), from the hind limb. In vitro, some adult muscle fibers isolated from wild-type mice respond to perfusion with chlorantraniliprole (intracellular calcium concentration increase), but at higher concentration range than bee cells (Fig. 6A and B, 35 μ M, a results representative of 5 cells). In muscle fibres isolated from a malignant hyperthermia mouse model (MH-RyR), chlorantraniliprole not only induced calcium transients, but cell contractions as well (Fig. 6C and D). Finally, in fibers from the MH-RyR model, cyantraniliprole 35 μ M also induced calcium transients and contraction (Fig. 6E).

Discussion

Insecticide overuse in agriculture is involved in insect decline². Although substantial evolutions have been made over the years to better estimate insecticides' side effects on honey bees, data gaps are still repeatedly identified by safety agencies, which also point out the need to explore toxicity on other beneficial insects^{31,36–41}.

Our LD₅₀s in the hundreds of nanomolar range confirm the high toxicity of cyantraniliprole (active substance) to honey bees (LD₅₀ at 24h after exposure). Indeed, insecticides leading to LD₅₀ below 2 000 ng/bee are traditionally described as 'highly toxic to bees'⁴². Furthermore, our data emphasize that the dorsal thorax route, which is mandatory in OECD test 214³¹, is the least sensitive zone and thus the least protective intoxication scenario (in our case, LD₅₀ was 885 ng/bee when the thorax was exposed, whereas other routes gave LD₅₀s

between 80 and 148 ng/bee). The actual toxicity of cyantraniliprole has thus been underestimated in the draft assessment report (D.A.R.) when using this least protective route of exposure⁴³. Furthermore, in the D.A.R., contact toxicity experiments on the active substance were restricted to a quite narrow range for adult bees (24–93 ng/bee) owing to the low cyantraniliprole solubility in water (section IIA 8.7.1/02 of the D.A.R.). Experimenters working for the company have chosen to use water as a vehicle instead of acetone, the latter being traditionally used in OECD 214 tests for hydrophobic compounds. In their case, the active substance applied on the dorsal thorax gave an LD₅₀ of 336 ng/bee (a value that we derived from mortality data at 72h available in the D.A.R., using a logistic extrapolation). In the present work, we used acetone in order to explore a wider range of doses, making it possible to efficiently measure the LD₅₀. Our data thus brings a useful complement to the imprecise endpoint depicted in the D.A.R. ('LD₅₀ > 94 ng/bee') and reported in toxicology databases (e.g., in the Pesticide Properties DataBase, PPDB). Another methodological weakness in the D.A.R. contact test must be pointed out: the volume of water deposited on the dorsal thorax was 5 µl, an amount difficult to fully deposit on the thorax and that hardly evaporated before the adult bees woke up from anaesthesia, with a possible loss of substance when bees moved around in the cage. We used 1 µl of acetone, efficiently applied and readily evaporated. In the same D.A.R., three candidate commercial formulations led to contact LD₅₀ of 256 ng/bee, 623 ng/bee, and 3188 ng/bee at 72h (values extracted with a logistic fit of mortality data described in the D.A.R.). The excessive volume mentioned above for the test with the active substance was less likely to arise in contact tests depicted in the D.A.R. with the formulated products (sections IIA 8.7.1/08, IIA 8.7.1/03, IIA 8.7.1/01), where 2 µl droplets were used. Water was, in this case, containing adjuvants, allowing higher solubility range for cyantraniliprole⁴⁴, and eventually giving nominal doses up to 16 000 ng/bee. In the literature, the high toxicity of cyantraniliprole

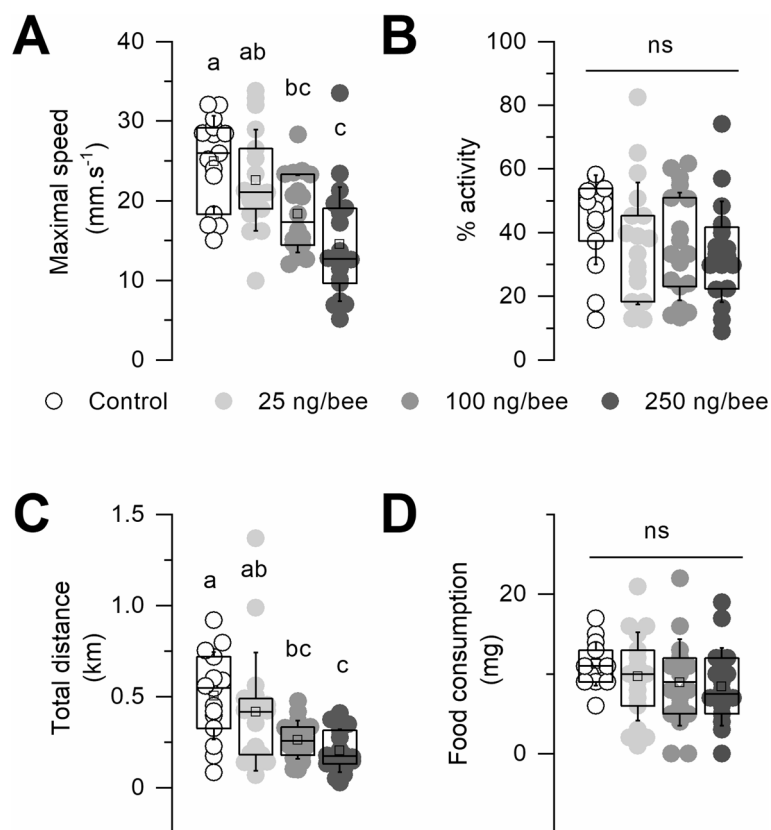


Fig. 3. Diminution of bees' performances after acute thoracic exposure to doses of cyantraniliprole. **(A)** Individual maximal speed of control bees (open dots, $n = 15$ bees) during the 21 h observation period. Maximal speed of bees exposed to 25 ng (light grey, $n = 18$), 100 ng (medium grey, $n = 18$) and 250 ng/bee (dark grey, $n = 18$). Groups move at significantly different speeds (Anova, $F = 9.57617$, $p < 0.001$). Different letters show significant differences. Speed decreases as the dose increases. Controls are different from 100 and 250 ng ($p = 0.0149$ and $p < 0.0001$, respectively, Tukey test). Group 25 ng is different from group 250 ng, $p = 0.0011$. **(B)** Percentage of time each bee was active in the different modalities. Groups are active during similar durations (Kruskal-Wallis, $p = 0.2345$, ns = not significant). **(C)** Total distance covered by individual bees after exposure decreases with the received dose. Controls are different from 100 and 250 ng (Dunn's test, $p = 0.0342$ and $p < 0.0001$, respectively). Group 25 ng is different from 250 ng ($p = 0.0416$). Food consumption was not significantly affected (panel D, Anova, $F = 1.0633$, $p = 0.3709$), nor was percentage of activity, whereas maximal speed and total distance were significantly decreased by the insecticide. Square symbols show the mean values. Box and whiskers show 25th, 50th, 75th percentiles and standard deviation to the mean. Boxplots displaying different letters in a same panel are significantly different from each other.

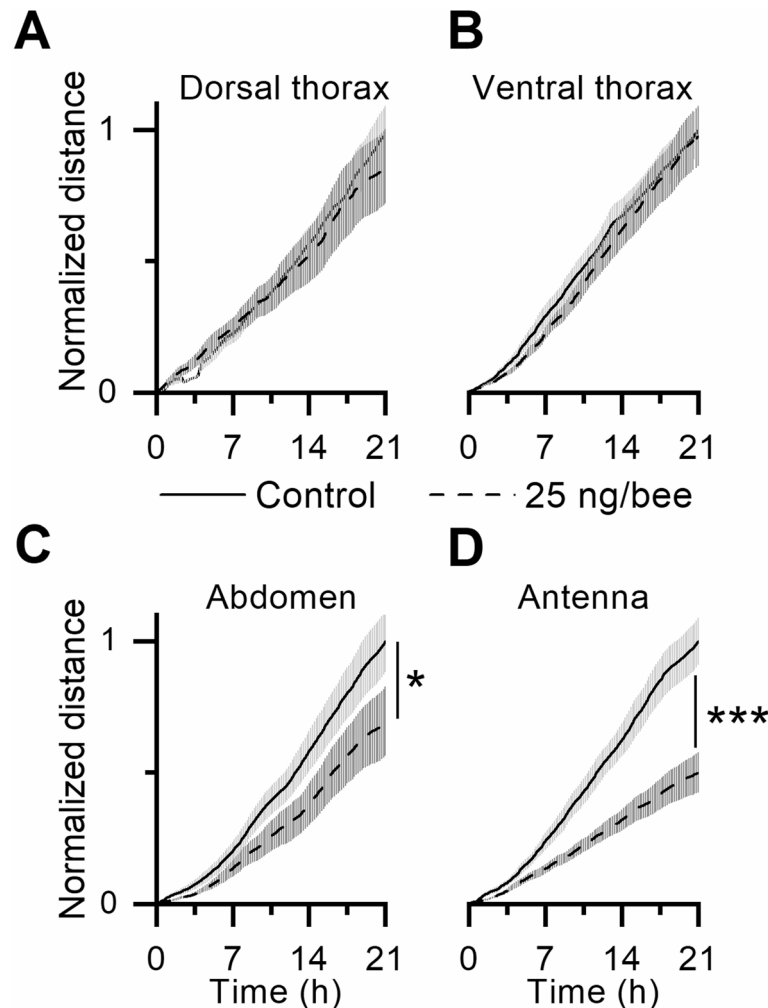


Fig. 4. Differential effects of a low cyantraniliprole dose in honey bees. For an exposure zone located either on the dorsal thorax (A) or the ventral thorax (B), the mean cumulated distance ($\pm$ S.E.M.) covered in 21 h by bees exposed to the low dose 25 ng/bee (sublethal, dashed line, $n = 18$ bees) superimposes to the cumulated distance covered by control bees (continuous black line, $n = 15$ bees). (C) When the dose 25 ng/bee was applied on the dorsal abdomen (close to the heart), the distance was significantly decreased at 21h (Mann–Whitney test, $p = 0.033$). (D) Antenna was the most sensitive zone, with a decrease by 51% of the cumulated distance ($p = 0.0001$). Asterisks show statistical difference.

(active substance) to honey bee larvae was also shown, both after acute or chronic oral exposure (47–64 ng/larvae, Kim et al. 2022).

Our work also helps filling data gaps identified in the estimation of cyantraniliprole effects on honey bee behaviour. Besides LD_{50} measurements, we assessed the behavioural effects of low doses of cyantraniliprole on honey bees. Two zones were found more susceptible: the antenna and the dorsal abdomen. Future experiments will determine whether cyantraniliprole toxicity via the dorsal abdomen is a consequence of cardiotoxic effects, as we recently demonstrated for chlorantraniliprole, which releases Ca^{2+} from intracellular stores, blocks voltage-gated calcium channels, and causes cardiac defects¹³. Cardiac impairment could indeed lead to a global decrease in performance, and a recent study showed that ingestion of cyantraniliprole (commercial formulation) impairs walking and take-off activities of wild bees⁴⁵. Concerning the higher antennal toxicity that we identified, it will be interesting to characterize antennal function after exposure, for instance with sucrose sensitivity tests or antennal responsiveness to odors with electroantennography⁴⁶. Whereas another diamide insecticide, flubendiamide, can anarchically release calcium in honey bee antennal neurons¹², neuronal effects remain to be explored with cyantraniliprole.

Our results show that cyantraniliprole can induce anarchic intracellular calcium mobilization in both bee and mouse adult muscle cells. To our knowledge, cyantraniliprole has never been functionally tested on RyRs from adult mouse skeletal muscle fibers. Our data thus provide the first evidence of cyantraniliprole action both in adult mouse skeletal muscle cells and adult bee muscle cells, both constitutively expressing RyRs. Whereas adult mouse *fdb* fibers express predominantly the RyR1 mammalian isoform, bee muscle fibers express an insect isoform that shares only ~45% homology with the mammalian RyR1^{22,24,47}, which may explain the molecular

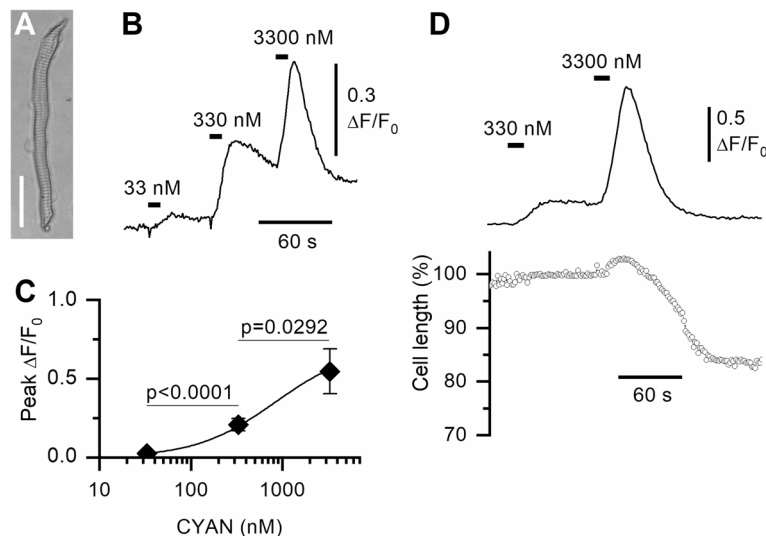


Fig. 5. In vitro exposure of bee skeletal muscle cells to cyantraniliprole. **(A)** Phase contrast image of an in vitro skeletal muscle fiber, attached to the bottom of a Petri dish (cell width 20 μm). **(B)** Cytoplasmic calcium increase measured in one cell as a function of time in response to sequential extracellular perfusion with 3 cyantraniliprole concentrations (10 s each, back in control solution in between). **(C)** Average calcium transients' maximal value (expressed as Peak $\Delta F/F_0$, $\pm$ S.E.M) obtained in the same conditions as in panel A ($n = 15$, 14 and 8 cells, for the 3 concentrations, respectively). P values from Mann–Whitney tests are indicated. **(D)** An example illustrating that in some cells, at the highest concentration, calcium transients (upper panel) could trigger contraction (cell slowly shortened by $\sim 20\%$ after exposure to 3300 nM, lower panel). Calcium transients' amplitude were only measured in the absence of contraction or right before any sign of contraction.

basis for the differential efficiency we identified in cells from the two species. Our study also provides the first evidence for chlorantraniliprole effects in intact adult mouse skeletal muscle fibers, further documenting its

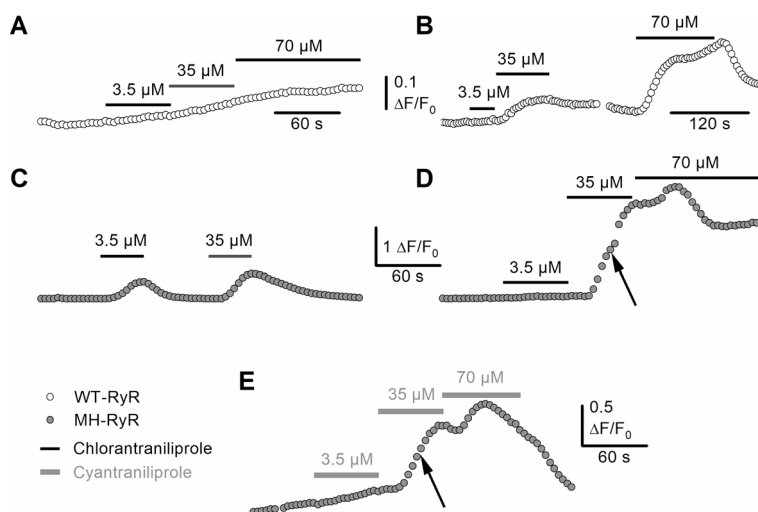


Fig. 6. Diamides can induce an anarchic cytoplasmic calcium increase in skeletal muscle fibers from wild-type and MH adult mouse. **(A and B)** Chlorantraniliprole, the first generation anthranilic diamide (which only differs from cyantraniliprole by substitution of the cyanide radical with a chloride atom) can induce calcium increase in a wild-type mammalian muscle cell (WT-RyR, white dots), in the absence of any sign of contraction ($n = 5$). The effect on intracellular calcium level is observed at higher concentrations than on bee muscle cells. Two example cells are given, one with a calcium transient (panel B, $n = 2$) and the other showing slow increase of intracellular Ca^{2+} concentration (A, $n = 3$). **(C)** in a mouse model of malignant hyperthermia (carrying mutation in the *ryr* gene, the MH-RyR mouse, grey dots), chlorantraniliprole induces a clear calcium increase in this cell at 3.5 and 35 μM . **(D)** another example where chlorantraniliprole 35 μM is inducing a contracture (the arrow indicates the start of cell contracture, representative of 5 MH-RyR cells). **(E)** Cyantraniliprole also induced large cytoplasmic calcium increases and contraction in 3 MH-ryr mouse cells (the arrow indicates the start of cell contracture). A and B panels share the same Y axis scale bar.

effects on skeletal muscle fibers and cardiomyocytes from adult bees^{11,13}. Chlorantraniliprole efficiency on immature mouse muscle cells was previously quantified, with an EC_{50} of 14 μM ⁴⁸, but to our knowledge, an EC_{50} value is not available yet for cyantraniliprole in these cells (C2C12 cell line) or any other mammalian muscle cells. In an early study, cyantraniliprole failed to stimulate [(3)H]ryanodine binding in rabbit muscle samples, whereas it stimulated binding in honey bee muscle samples, suggesting that cyantraniliprole interacted with bee RyRs but not with rabbit RyRs⁴⁹. In this early publication, the direct binding of cyantraniliprole on rabbit RyRs was not studied, but [(3)H]chlorantraniliprole was not able to bind rabbit RyRs ('no significant specific binding was observed'), while it clearly bound bee RyRs (12 ± 4 fmol/mg bee muscle). These initial results were later contradicted by functional RyR studies showing that chlorantraniliprole interferes with rabbit RyR function^{26–28}. Our results on bee muscle cells concur with an earlier study on insect RyRs, in which both cyantraniliprole and chlorantraniliprole stimulated ryanodine binding in muscle samples from a dipteran (the fly *Musca domestica*), a hymenopteran (the domestic bee, *Apis mellifera*), and lepidopterans (two moths from the noctuidae family, *Agrotis ipsilon* and *Heliothis virescens*)⁴⁹.

Our results further document alterations of insect RyRs by cyantraniliprole, but in a cell preparation where channels are naturally expressed in their normal muscular cytoplasmic environment, whereas in previous studies^{50,51}, RyR expression was forced in heterologous systems HEK293 (cell line derived from human embryonic kidney) or Sf9 (cell line obtained from ovarian insect tissue). In these heterologous expression systems, EC_{50} for cyantraniliprole was between 12 and 250 nM for aphid, fly, and moth RyRs. A recent study also documented cyantraniliprole effects on purified vesicles of sarcoplasmic reticulum from the thoracic muscles of another moth species (*Mythimna separata*), with an EC_{50} between 410 and 790 nM, the concentration–response reaching a plateau between values 1000 and 10,000 nM⁵². Again, in this context, RyRs carried by purified vesicles may keep a natural in situ environment on their luminal side, but not on their cytoplasmic side. Diamide's chronic use was shown to induce resistant insect populations, including in certain moth species⁵³, but to our knowledge, such resistance has never been identified in honey bees. A target resistance to diamides was mapped at amino acid number 4946, a glycine, in the moth *Plutella xylostella* RyR, located in the C-terminus region, between transmembrane domains TM4 and TM5^{53,54}. The mutation decreased the field sensitivity of the moth *Plutella xylostella* to chlorantraniliprole by a factor of 200⁵³. The mutation also decreased the *P. xylostella*-RyR sensitivity to chlorantraniliprole by > 200-fold in an in vitro heterologous expression system⁵⁴. The *Apis mellifera* RyR protein sequence is ~80% identical to those of insects considered as pests⁵⁴. The glycine residue is present in both *Apis mellifera* and several wild bee species, which are 95–98% identical with each other^{22,23}, and to our knowledge, a mutation that would confer less sensitivity of bee RyRs and resistance to diamides has never been described in any bee species.

In addition to testing chlorantraniliprole on adult mouse muscle fibers, we investigated the effects of diamides in muscle fibers from the first murine model of Malignant Hyperthermia, which carries a heterozygous RyR1^{Y522S/+} mutation⁵⁵. If not treated immediately, MH can be a lethal condition in humans. A hallmark of MH is an increased susceptibility to heat stress and volatile general anaesthetics, and the RyR1^{Y522S/+} model shows a stronger susceptibility to both of these stressors. Increasing temperature over 35 °C induced sustained basal contraction in RyR1^{Y522S/+} mice soleus, while temperatures ≥ 41 °C were required to induce basal contractions in WT soleus. Caffeine-induced contraction in soleus muscles from both adult WT and RyR1^{Y522S/+} mice, but the latter were more sensitive to caffeine at 25 °C, with EC_{50} s of 19 mM and 5 mM, respectively. Our results show that muscle fibers isolated from the *flexor digitorum brevis* dissected from RyR1^{Y522S/+} adult mice also show a higher sensitivity to chlorantraniliprole than their WT controls. Other murine models of MH exist, for which an exploration of susceptibility to diamide would be important. The majority of RyR1 human mutations linked to MH are clustered in two regions of the cytoplasmic domains of RyR1 (amino acids 35 to 614 and 2129 to 2458), and another cluster of mutations is found near the carboxyl terminus (4637 to 4973), as reviewed in⁵⁶. Available mouse MH models include animals with mutations in region 1 (R163C and Y522S), region 2 (G2435R and R2509C), or region 3 (T4826I), leading to hyperthermia^{55,57,58}. The mouse model that we used carries a mutation Y522S in a region of RyR which is not directly involved in anthranilic diamides binding, since they are suspected to bind in the C-terminus transmembrane region encompassing amino acids 4111–5084²⁸. Furthermore, this mutation is not located in the suspected binding region for flubendiamide, a phthalic-based diamide (residues 183–290 of the N-terminus region). Using the ryanodine binding assay on SR vesicles prepared from mouse limb muscle, Truong & Pessah showed evidence for an increased chlorantraniliprole susceptibility of RyRs carrying an MH-triggering mutation (T4826I) at a low calcium concentration (significantly fourfold lower EC_{50} and 300-fold smaller 'lowest observed effect concentration'). They also showed a significant twofold lower EC_{50} for R163C-RyRs²⁸. More importantly, wild type RyRs, T4826I-RyRs and R163C-RyRs exposed to chlorantraniliprole lose their ability to close at high calcium concentration (1 mM), this mechanisms being essential to prevent an excessive positive feedback and uncontrolled and deleterious calcium liberation from SR. In the future, it would be interesting to explore the sensitivity to cyantraniliprole and chlorantraniliprole of intact mouse muscle fibers from those two MH mouse models, working at physiological body temperatures.

Our findings demonstrate that cyantraniliprole exerts pronounced toxicity on bees, particularly following exposure of the antennae or dorsal abdominal regions. These observations suggest that olfactory processing and cardiac activity may be especially vulnerable to this new-generation diamide insecticide, even at sublethal concentrations. In addition, cyantraniliprole disrupts calcium homeostasis in mouse muscle fibers, although at higher concentrations than those affecting bee muscle tissue. Data obtained from muscle fibers of the MH mouse model further emphasize the need for comprehensive studies on the sensitivity of naturally mutated mammalian ryanodine receptor isoforms, RyR1 and RyR2, to diamide compounds. Such investigations are essential to determine whether individuals carrying pathogenic mutations exhibit increased susceptibility within both skeletal and cardiac muscle systems.

Methods

Honey bees

Honey bees (*Apis mellifera*) were obtained from hives belonging to the experimental apiary of the *Bees & Environment* Research Department, located in Avignon. Newly emerged bees were collected from a frame with developing brood and placed overnight in an incubator. Bees were placed in aerated plastic cages with 1 feeder containing water and 1 feeder containing commercial Candi sugar paste (Apifonda, Icko-Apiculture).

Classical toxicology

Bees were anaesthetized in an anesthesia chamber (30 s exposure to a controlled CO₂ volume) and placed on an ice-refrigerated surface to maintain anesthesia during contact exposure to a 1 µl droplet of acetone solution containing cyantraniliprole at concentrations 0 (control), 25, 100, and 250 ng/µl. In four separate experiments, bees were exposed either via dorsal thorax (the traditional route of exposure in method OECD 214) or ventral thorax, dorsal abdomen, or left antenna. Each bee was then transferred into a clean Petri dish (diameter 50 mm, depth 15 mm; Thermo Fisher, Rochester, NY). A feeder filled with Candi sugar paste ad libitum was placed on the right lateral region of each Petri dish. Each Petri dish was inserted in a hole drilled in a plate held vertically in an incubator (30 °C, red light). Survival was monitored for 72 h. Another classical mortality test was performed, in which bees were introduced into collective cages after exposure to the dorsal thorax (10 per cage), as depicted earlier⁵⁹. Cyantraniliprole doses ranged from 10 to 6000 ng/bee (30 × 10 bees per dose). Bees were provided with food and water ad libitum and kept at 29 °C for 72 h.

A logistic function was fitted in Origin 2023 (OriginLab, Northampton, USA) to dose-mortality data for each exposure zone to extract the median lethal dose (LD₅₀). The logistic function had the following equation: %Mortality = %M_{min} + (%M_{max} - %M_{min}) / (1 + exp(-slope * (log(dose) - log(LD₅₀)))), where %M_{min} and %M_{max} were minimal and maximal mortality, respectively.

Locomotor performances after exposure to low insecticide doses

After bees had been transferred to Petri dishes as described above, locomotor performances were assessed in these individual arenas. A constant dark cycle was chosen to better mimic lighting conditions experienced by newborn bees in the first day of their life. Arenas were placed vertically 4 cm in front of a red screen (a computer monitor with colour settings set to red only, a color that bees do not see and thus mimicking darkness). A webcam (Microsoft LifeCam, 640 × 480 pixels) placed 40 cm away from the screen allowed for the monitoring of bees' displacements. The monitor, the plastic tray, and the webcam were enclosed in an opaque climatic chamber (Model #3744, Forma Scientific Inc., Marietta, Ohio) to maintain constant temperature (30 °C) and avoid the influence of daylight variations. The webcam was operated with Image J software⁶⁰, through a custom-made plugin⁵⁹. In brief, the center of mass of each bee was detected online during 21 h, allowing the measurement of its instantaneous speed at a frequency of 20 Hz. Observation always started between 9:30 and 10 a.m. A speed threshold of 3 mm/s was set as the limit for an actual walking activity. The plugin was configured to allow an online measurement of the average speed and distance covered per 10-min bin. These values were afterwards used to calculate the % of time each bee was active, its maximal speed, and the total distance it covered during the whole 21-h observation period. The weight of food ingested was calculated from measures taken at the beginning and at the end of the experiment.

In vitro calcium imaging on bee muscle fibers

Bee skeletal muscle fibers were obtained following a procedure described previously¹⁵. In brief, bees were chilled (~4 °C) until lethargic immobility and then killed by decapitation. Tibias from the third pair of legs were dissected in a Tyrode's solution without calcium (in mM: NaCl 140, KCl 5, MgCl₂, HEPES 10, pH 7.2), cut longitudinally, and pinned open. Tibias were bathed for 20 min in a dissociation solution at 37 °C (Tyrode's solution without calcium, but containing four enzymes: collagenase type 1, trypsin, papain, and pronase at concentrations 0.5, 1.5, 1.5, and 1 mg/ml, respectively). Isolated fibers were obtained by mechanical trituration in a Petri dish. Cells were loaded with the membrane-permeant fluorescent calcium indicator Fluo-3 AM (10 µM, 30 min), and fluorescence was measured as described earlier¹⁵. Briefly, a Qicam camera operated with QCapture Pro (QImaging, Canada), allowed for cell image capture at frequency of 1 Hz. Cell fluorescence was measured offline with Image J (NIH, USA) on a region of interest chosen in an area of the cell well attached to the bottom of the Petri dish. Fluorescence values were expressed as ΔF/F₀ signals, F₀ being the resting (or baseline) fluorescence at the beginning of recording (in control solution), and ΔF the change in fluorescence from baseline. A gravity-driven perfusion system (polyethylene tubing, rate 100 µl/min) controlled via pinch valves (Valvelink, Automate Sci, USA) was automatically operated via the WCP software (J. Dempster, University of Strathclyde) using an A/D board (National Instruments, USA). Extracellular solution around a single cell was quickly and efficiently changed with this system. A stock solution of cyantraniliprole was prepared (10 mM in DMSO) and diluted in a Tyrode solution with 2 mM calcium. For experiments on bee cells, cyantraniliprole was used at 33, 330 and 3300 nM starting with the lowest concentration (10 s each, back in control solution in between). The final DMSO concentration was 0.1% in all solutions. All measurements were carried out at room temperature (20–23 °C).

In vitro calcium imaging on mouse muscle fibers

Mouse experiments followed the guidelines of the European Community (86/609/EEC). The experimental protocol was approved by the institutional Animal Care Committee of the University of Debrecen (3–1/2019/DEMAB). The study is reported in accordance with the ARRIVE guidelines for animal experiments. The mice were housed in plastic cages with mesh covers and fed ad libitum with pelleted mouse chow and water. Room illumination was an automated cycle of 12 h light and 12 h dark, and room temperature was maintained within the range 22–25 °C. The intracellular calcium concentration measurements were carried out on enzymatically

isolated single skeletal muscle fibers from the *flexor digitorum brevis* (fdb) muscle of adult (3–6 month old) mixed gender control C57/BL6 and RyR1Y522S (MH-RyR) mice. This strain was kindly provided by Janos Almasy (Department of Physiology, Debrecen), who got it from Susan L. Hamilton (Baylor College of Medicine) and Werner Melzer (Ulm University). Mice were anesthetized using the drop method for an isoflurane concentration below 2%. After cervical dislocation, *flexor digitorum brevis* from the hind limb was dissected manually under a transmitted light microscope using thin forceps and fine precision surgical scissors. Muscles were manually dissected in calcium-free Ringer's solution (containing in mM: 136 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 10 glucose; pH 7.2). Single muscle fibers from fdb were enzymatically dissociated in media containing 0.2% Type I collagenase (Sigma) at 37 °C for 45–50 min. After digestion, the FDB muscles were mechanically dissociated to release single fibers with gentle trituration in normal Ringer solution (same as above but supplemented with 2.6 mM CaCl₂). The isolated fibers were stored at 4 °C in a refrigerator until use. Only fibers with clearly visible striations, no swelling, or surface membrane damage were selected for experiments. Mouse cells were loaded for 30 min with the cell-permeant calcium indicator Fluo-8 AM and imaged with a laser scanning confocal microscope Zeiss LSM 5 LIVE (Zeiss, Oberkochen, Germany), following a procedure described earlier⁶¹. Series of 1024 × 1024 pixel images were captured at 0.25 Hz. The pixel size was 0.65 µm. Cell fluorescence was expressed as $\Delta F/F_0$ signals after quantification offline with ImageJ. A stock solution of cyantraniliprole was prepared (14 mM in DMSO) and diluted in a Tyrode solution with 2 mM calcium. For experiments on mouse cells, cyantraniliprole was used at 3.5, 35 and 70 µM (final DMSO 0.1, 0.25, and 0.5%, respectively). Chlorantraniliprole stock solution was prepared at 7 mM in DMSO and diluted in a Tyrode solution with 2 mM calcium. Concentrations 3.5, 35, and 70 µM were used on mouse cells (final DMSO 0.1, 0.5, and 1%, respectively).

Statistical analysis

Data are given in mean ± S.E.M. (standard error of the mean) in the text of the Results section and in Figs. 4 and 5. In addition to means, quantiles and standard deviation are indicated in Fig. 3. Data normality was assessed, and either Kruskal–Wallis and Dunn's post-hoc test (Fig. 3B, C), Anova and Tukey test (Fig. 3A and D) or Mann–Whitney test (Fig. 4 and 5C) were performed, accordingly. Fisher exact test was used to assess significance in mortality data (Fig. 1B and Supplementary Fig. 1). A *p* value < 0.05 was considered significant. Statistics were performed under Graphpad Prism 10 and Origin 2023 (OriginLab, Northampton, USA).

Data availability

All data generated or analyzed during this study are included in this article.

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Author contributions

Designed the experiments: M.C., C.C., P.S. Performed the experiments and analyzed the results: M.C., C.C., and P.S. Wrote the paper: M.C., C.C., P.S., L.S., A.K., J.M., and P.C. All authors reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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