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Choice of vehicle affects pyraclostrobin toxicity in mice

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Abstract

Pyraclostrobin is a strobilurin fungicide that inhibits mitochondrial complex III of fungal and mammalian cells. In toxicity studies that were used to estimate the safety factor, pyraclostrobin was added to animal feed or to aqueous vehicles. However, foods containing residues of pyraclostrobin and other strobilurin fungicides (azoxystrobin, trifloxystrobin, fluoxastrobin) are frequently prepared in vegetable oil prior to human consumption. The primary objective of this study was to determine if pyraclostrobin dissolved in an oil-based vehicle had adverse health outcomes in mice when compared to aqueous-based vehicles. We found that pyraclostrobin does not fully dissolve in aqueous methyl cellulose (MC) or carboxymethyl cellulose (CMC), two vehicles used in industry-sponsored toxicity studies, but does fully dissolve in corn oil. Moreover, C57BL/6 mice receiving pyraclostrobin in corn oil displayed adverse health outcomes, including loss of body weight, hypothermia and diarrhea at lower doses than when added to feed or to aqueous vehicles. Our data suggest that previous studies underestimated the true toxicity of pyraclostrobin in mammals. Additional toxicity tests using oil-based vehicles are recommended to verify current safety recommendations for strobilurin fungicides.

Keywords

Toxicity; preclinical; pyraclostrobin; mice; strobilurin fungicides

1. Introduction:

Given the rapid pace of new pesticide development and deployment in the environment, there is an ongoing need to develop better screening techniques to qualify potential health risks. To accelerate the pace at which candidate environmental risks for neurological

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Conflict of Interest Disclaimer

The authors have nothing to disclose.

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disorders are identified, our group recently exposed cultured mouse cortical neurons to 294 environmental-use chemicals, then profiled gene expression using RNA-seq (Pearson 2016). We identified a group of chemicals that upregulated many of the same neuroimmune genes and downregulated many of the same synaptic genes that are affected in individuals with autism and neurodegenerative disorders. Chemicals in this group included rotenone, a pesticide linked to Parkinson's disease risk (Tanner 2011; Sherer 2007), two strobilurin fungicides (pyraclostrobin, trifloxystrobin), fenamidone, and famoxidone. Fenamidone, famoxidone, and strobilurin fungicides inhibit mitochondrial complex III and, as we found, generate reactive oxygen species (ROS) and destabilize microtubules in neurons. Usage of these fungicides has increased on a diversity of food crops since their EPA registration in 2000 (Pearson 2016). Additionally, a related strobilurin (azoxystrobin) is now used in building materials (wallboards), posing a new potential vector for exposure via dust.

In researching toxicity data on these fungicides, we took note of the Lowest Observed Adverse Effect Levels (LOAEL) and No Observed Adverse Effect Levels (NOAEL) reported by multiple governmental regulatory agencies (Bartholomaeus 2003; Australian Pesticides and Veterinary Medicines Authority 2003; Moore 2011). These number were established based on studies in rodents that added strobilurins directly to animal feed (Mellert 1998, 1999) or that added strobilurins to aqueous methyl cellulose or carboxymethyl cellulose for oral gavage (Mellert 2002). However, pyraclostrobin is poorly soluble in water: 1.9 mg/L at 20 deg C (Tomlin 2004). Looking first at acute oral dietary toxicity reports, the LOAEL for rats (indicated by significant weight loss after consuming pyraclostrobin on chow) was 1000 mg/kg/day (Bonner 2001). A one-week study that used oral gavage and pyraclostrobin-containing chow (18 mg/kg/day) failed to find treatment-related effects in male mice (Mellert 2002). A subchronic mouse study (lasting 3 months) found that the LOAEL after pyraclostrobin was added to chow (indicated by body weight changes after 3 months of daily feeding) was 30-40 mg/kg/day (Mellert 1998, 1999).

Today, strobilurin fungicide residues are found on foodstuffs, including spinach and kale, which are often sautéed in vegetable oils or consumed with oil-based dressings. To account for the potential increased bioavailability of pyraclostrobin in oil-soluble vehicles, we sought to examine the solubility and acute toxicity of pyraclostrobin in mice using an oil-based vehicle. Our study was designed to more accurately model additional types of oral exposure in mice using previously established NOAEL levels of pyraclostrobin stated in acute and subchronic oral toxicity reports.

2. Materials and Methods

2.1 Animals

Male C57BL/6 (Jackson Laboratories) mice (age 3-6 months) were used for all experiments. Mice were group-housed under a 12-h light, 12-h dark cycle (lights on at 07:00 h) in a temperature-controlled environment (20 ± 1 °C) with *ad libitum* access to food (Envigo Teklad 2920) and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and in accordance with NIH guidelines.

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2.2 Chemicals

Pyraclostrobin (ethyl N-{2-[1-(4-chlorophenyl)-1H-Pyrazol-3-yloxy-methyl]phenyl}(N-methoxy)carbamate; ≥ 98.0 purity; Sigma-Aldrich) was mixed in 0.5% aqueous methyl cellulose (MC; Sigma), 0.5% carboxymethyl cellulose (CMC; Sigma), or corn oil (CO; Ward's Science) for up to 3 hours at 45°C in a water bath sonicator.

To compare the amount of dissolved pyraclostrobin in our dosing solutions, we performed an analysis of pyraclostrobin in stock solutions by Liquid Chromatography/Mass Spectrometry (LC/MS). We also tested our control corn oil for a second strobilurin, azoxystrobin. Due to the high concentration of the dosing solutions, 10 μL of each solution was diluted in 10 mL LC/MS grade water (Honeywell Burdick Jackson, Muskegon, MI) prior to analysis. For analysis, 10 μL of the diluted stocks was transferred to an LC/MS vial, spiked with 10 μL D_6-linuron (CDN Isotopes, Pointe Claire, Quebec, Canada) as an internal standard, and diluted to 1 mL with 1:1 LC/MS-grade methanol:water. Triplicate blanks were prepared identically using LC/MS grade water. Analysis was performed on an Agilent 1260 liquid chromatograph with 6460 triple quadrupole mass spectrometric detector with positive mode atmospheric pressure chemical ionization (APCI) source (Agilent, Wilmington, DE). Separation was achieved on a Phenomenex (Torrance, CA) 50×2 mm 2.5 μm particle size Luna C-18 column at 40°C with the following gradient program of 10 mM formic acid ("A"):10 mM formic acid in methanol ("B"): A:B 70:30% from 0-0.2 min, to 99% B at 2 min held until 6 min, followed by re-equilibration to initial conditions. Flow rate was 0.3 mL/min and injection volume was 15 μL. The APCI source and gas temperatures were held at 350°C and 325°C, respectively. Gas flow was 8 L/min, nebulizer pressure was 40 psi, the capillary current was (+)4500V and corona set was set at (+)4 μA. Compounds were detected with multiple reaction monitoring of the transition shown in Supplementary Table 1 using authentic standards (azoxystrobin: Santa Cruz Biotechnology; pyraclostrobin: Sigma Aldrich).

For subsequent animal studies, doses of pyraclostrobin were selected based on previous toxicity acute oral dose studies that established NOAEL doses in rodent models (Mellert, 1998; 1999; 2002). 0, 10, 100, or 400 mg/kg pyraclostrobin were administered by oral gavage (*po*) to mice immediately before observation or behavioral testing. Control groups received vehicle (corn oil, methyl cellulose, or carboxymethyl cellulose) alone. 400 mg/kg, the highest dose of pyraclostrobin, was selected because it was less than 50% of the reported LOAEL for acute dosing using rats.

2.3 Dosing and health monitoring

Prior to dosing, baselines for body weight and core temperature (via rectal thermometer) were collected. Mice were then gavaged and immediately transferred into a clean cage with wax paper to facilitate collection of fecal boli. At three hours after dosing, body weight, core temperature, and fecal boli were collected, and mice were transferred back into their home cage. Fecal material collected on wax paper flooring from the test chamber was weighed to establish a "wet weight" and then dried at room temperature for 48 hours, as previously reported (de Theije 2014). Material was weighed at this time to establish a "dry weight" and used to determine, via a simple percent change formula, the amount of water contained in

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collected fecal material. Measures of general health were also collected at hour 24, including body weight and core temperature. All measures were collected by a blinded experimenter; animals were randomly assigned to dosing groups and subsequently gavaged by a second person.

2.4 Assessing locomotor activity

To assess the effects of pyraclostrobin on motor activity, a separate cohort of naïve male C57Bl/6 mice (n=20; 3-4 months old) were trained during their light cycle on a wheel-running assay for two weeks prior to dosing as described (Cobos 2012). At the start of training, mice were placed in a cage with a large metal wheel and allowed to run for 1 hour. After 4 one-hour sessions, a subset (n=16) of high-performing animals was selected and subsequently trained for 2 hours a day for the rest of the two-week training period. To habituate mice to oral gavage, animals received oral doses of corn oil alone (10 mg/kg, *po*) 1 hour prior to wheel-running on the last 3 training days. We counted the number of wheel rotations over a 2 hour period during these last 3 days using the computer program Graphic State (Coulbourn Instruments), and the average of these three measurements was used as a baseline running score. The following day mice were dosed with pyraclostrobin (100 mg/kg or 10 mg/kg, *po*) 1 hour prior to wheel running. Mice were then tested for up to 3 days after dosing and wheel rotations were recorded over a 2 hour period each day. Percent change in wheel running activity was calculated for each post-dose time point. Measures of general health were collected at hour 3, 24, and 48 by an experimenter (G.S.) sufficiently blind to dose status.

2.5 Statistical analysis

All reported statistical findings were calculated using Systat (v.13.1) with a criterion $\alpha = 0.05$. All graphs were made using Graphpad Prism (v.7.0a). Baseline behavioral outliers were identified (Studentized residual >3.0) and removed prior to final analysis ($n \leq 2$ cases/measure; Supplementary Table 2). Data were analyzed using one-way ANOVAs after determining normality of experimental data (Anderson-Darling test), followed by Bonferroni post hoc tests to determine group differences. In order to provide further context, we graphed changes from baseline for all behavioral indices (Supplementary graphs 1-2), and included raw data from both experiments (Supplementary Table 2).

Weight loss and hypothermia observed in both cohorts were calculated as area under the curve using the trapezoidal rule, with respect to weight and body core temperature baselines (taken before compound dosing). Percentage of maximum possible decrease in both measures (% Maximum Decrease) was calculated for each mouse as compared to a hypothetical subject with the same pre-dosing baseline thresholds and maximum body weight loss and body temperature loss at all post-dosing timepoints.

For wheel running data, a similar area under the curve using the trapezoidal rule was implemented, with respect to wheel running baselines. Percentage of maximum possible decrease in wheel running (% Maximum Decrease) was calculated for each mouse as compared to a hypothetical subject with the same pre-dosing baselines and zero wheel rotations at all post-dosing timepoints.

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3. Results

3.1 Pyraclostrobin solubility

Nearly all toxicology studies of mitochondrial complex III inhibitor fungicides, including pyraclostrobin, trifloxystrobin, picoxystrobin, and fenamidone, used aqueous cellulose-based vehicles to dose animals (Mellert 1999; Bartholomaeus 2003; Moore 2011). In our own hands, it was visually obvious that pyraclostrobin did not fully dissolve in aqueous methyl cellulose (MC) or carboxymethyl cellulose (CMC), even after extended (3 h) heating and agitation (Fig. 1a,b). After testing additional vehicles commonly used by regulatory agencies, however, we discovered that pyraclostrobin is fully soluble in corn oil (CO) following brief (15 minute) agitation (Fig. 1c). We also found that azoxystrobin, trifloxystrobin, picoxystrobin, and fenamidone were fully soluble in corn oil but were not soluble in cellulose-based vehicles (data not shown).

To quantify precisely how much pyraclostrobin dissolved in these dosing solutions, a compositional analysis of the dosing solution supernatants was performed using liquid chromatography/mass spectrometry (LC/MS-MS). Neither pyraclostrobin (Table 1) nor azoxystrobin (a second, related strobilurin used in agriculture; not shown) were detectable in the corn oil control, indicating that the corn oil vehicle was uncontaminated by these fungicides. Of the experimental dosing solutions tested, corn oil was the only solvent that fully dissolved pyraclostrobin at expected doses (Table 1). In contrast, the aqueous vehicles contained very little dissolved pyraclostrobin.

3.2 Acute effects of oil-based pyraclostrobin on adult mouse health

We next evaluated the acute effects of pyraclostrobin (400 mg/kg, *po*) dissolved in aqueous and oil-based vehicles on adult mice by monitoring several indices of general health including body weight, core temperature, and fecal boli. Contrary to previous acute dosing studies that used aqueous-based vehicles (Bonner, 2001), we found that a single 400 mg/kg pyraclostrobin dose dissolved in corn oil resulted in marked deterioration of murine health. Specifically, a one-way ANOVA analysis of calculated % weight decrease revealed that mice dosed with pyraclostrobin in corn oil (“CO + Pyra”) were the only treatment group that lost significantly more weight ($p<.001$) than vehicle controls ($F(5,33) = 17.1$, $p<.001$) 24 h following injection (Fig. 2a; Supp. Fig. 1). Similarly, an analysis of core temperature area under the curve data showed that CO + Pyra mice were the only treatment group that became hypothermic 24 h following injection, a significant reduction ($p=.007$) compared to control animals ($F(5,34) = 3.9$, $p< .01$; Fig. 2b).

Analysis of fecal boli revealed that mice receiving pyraclostrobin dissolved in corn oil or methyl cellulose had pronounced diarrhea. A one-way ANOVA analysis of fecal composition revealed that mice treated with either CO+ Pyra ($p= 0.008$) or MC + Pyra ($p= 0.029$) produced significantly more watery fecal boli than CO controls ($F(5,34)=10.6$, $p<.001$; Fig. 2c). Pyraclostrobin dissolved in methycellulose also caused diarrhea when compared to CO controls ($p<.05$), even though this vehicle only partially dissolves pyraclostrobin (Table 1). However, we note that animals treated with this vehicle failed to display any other signs of illness at any observation timepoint (Fig. 2 a,b).

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3.3 Establishing an exposure-response relationship for oil-based pyraclostrobin

Based on results from the first experiment, we formulated lower doses of pyraclostrobin in CO (0, 10 mg/kg or 100 mg/kg, *po*) in order to monitor changes in common health indices (including body weight, temperature, and fecal boli composition) in a second cohort of animals. We also tracked voluntary wheel running activity for up to 48 hr following gavage (Cobos 2012). We found that pyraclostrobin dissolved in corn oil significantly and dose-dependently affected animals across all measures. Beginning with wheel running activity, a one-way ANOVA analysis of our results revealed that 100 mg/kg pyraclostrobin significantly decreased voluntary running activity (p=.016) compared to oil-treated controls (F(2, 21) = 8.4, p<0.01; Fig. 3a; Supp. Fig. 2).

Similar to mice treated with 400 mg/kg of pyraclostrobin, 100 mg/kg CO + Pyra mice exhibited significantly reduced body weight for up to 48 hr (p<.001) compared to oil-treated controls (F(2, 24) = 17.9, p<.001; Fig. 3b). Likewise, mice treated with 100 mg/kg dose of pyraclostrobin showed significantly decreased core body temperature for up to 24 hr when compared to controls (F(2, 24) = 4.9, p=.016; Fig. 3c). Finally, a one-way ANOVA analysis of fecal boli composition revealed that pyraclostrobin caused diarrhea in a dose-dependent manner (10 mg/kg: p<.05 ; 100 mg/kg : p<.001) when compared to CO controls (F(2,24) = 25.3, p<0.001; Fig. 3d).

4. Discussion and Conclusions

Mitochondrial complex III inhibitor fungicides, including pyraclostrobin, have toxic effects on neurons in culture (Pearson 2016; Regueiro 2015). Strobilurin fungicides also promote triglyceride (fat) accumulation in mouse 3T3-L1 adipocytes, with pyraclostrobin showing supra-maximal effects relative to the positive control (Kassotis 2017). In our effort to replicate previous experiments, we noticed that the bulk of the toxicity tests cited in the regulatory literature dissolved pyraclostrobin in aqueous cellulose-based vehicles or added pyraclostrobin to animal feed (Mellert 1998, 1999, 2002; Bonner 2001). However, our inability to fully dissolve these fungicides in aqueous-based vehicles, even after extensive sonication and heating, lead us to test an oil-based vehicle.

We have shown that when fully dissolved in corn oil, pyraclostrobin doses as low as 100 mg/kg produced a significant reduction in body weight over a 48 h period after dosing (Fig. 3b; Supp. Fig. 2), as well as acute hypothermia and diarrhea in treated animals, albeit to a lesser extent than our initial 400 mg/kg dose (Supp. Fig. 1). These doses are significantly lower than established acute toxicity doses in rodents (>1000 mg/kg) and subchronic LOAEL doses (30-40 mg daily x 3 months) established by regulatory agencies (Moore 2011; Bartholomaeus 2003).

Furthermore, we found that mice displayed a significant reduction in running activity following exposure to 100 mg/kg dose of pyraclostrobin, offering additional evidence that these animals become acutely ill after being exposed to a single oral dose of these fungicides after the compounds are fully dissolved in an oil-based vehicle.

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Diarrhea was observed in mice gavaged with both our initial dose of pyraclostrobin dissolved in corn oil (400 mg/kg), as well as subsequent lower doses of compound (100 mg/kg and 10 mg/kg). Although mice given 400 mg/kg pyraclostrobin in methyl cellulose (MC) also showed signs of diarrhea, we note that these animals did not show concomitant signs of illness (body weight or body temperature changes), nor did they show sign of GI distress when MC was administered alone. Together, these results suggest that despite acute GI distress, the animals given pyraclostrobin in methyl cellulose were better able to tolerate exposure to the (relatively) lower concentration of chemical compound in our dissolved solvent (as demonstrated by LC/MS-MS analysis in Table 1). Our findings are consistent with previous subchronic dosing studies that reported gastrointestinal tract effects following the administration of higher doses of pyraclostrobin in aqueous vehicles or powdered directly onto rodent chow (Australian Pesticides and Veterinary Medicines Authority 2003; Mellert 1998, 1999, 2002; Bonner 2001; Moore 2011; Bartholomaeus 2003), although we find evidence for diarrhea at lower doses when pyraclostrobin is dissolved in corn oil. Since both methyl cellulose and carboxymethyl cellulose produce suspensions of undissolved pyraclostrobin particles, we cannot rule out the possibility that animals in our study received higher doses of pyraclostrobin than reported by our mass spectrometry analysis. Based on our findings, additional studies are needed, using solvents that fully dissolve strobilurin fungicides, to more carefully evaluate systemic toxicity.

The use of Complex III inhibitor fungicides, including pyraclostrobin, has greatly increased in conventional (non-organic) crop production since their registration in 2000 (Pearson 2016). Moreover, strobilurins were patented in 2012 for use in building materials, to make antifungal wallboards and insulation (Patent US 8138196 B2). Wallboards containing azoxystrobin (brand names, SporGard, OptiShield and Azotech) can be purchased in the United States at Lowes and other retailers. These boards are now being used in new construction wherever there is the potential for mold (bathrooms, basements, entire houses). Human exposure to these fungicides are likely increasing via food and dust in homes and buildings (Kassotis 2017). As such, new studies assessing novel routes of administration of pyraclostrobin and related strobilurins are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Pyraclostrobin and related strobilurins have poor solubility in aqueous solutions.
- Mice show adverse health outcomes at lower doses of pyraclostrobin dissolved in oil
- Previous toxicity studies using cellulose vehicles may underestimate exposure risk

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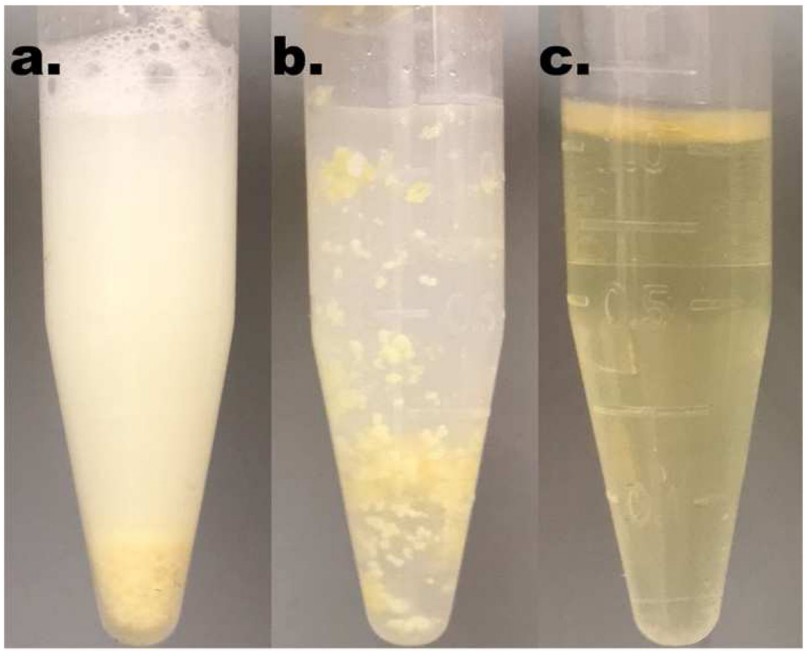

Fig. 1.
Pyraclostrobin (40 mg/ml) in **a)** methyl cellulose, **b)** CMC, **c)** corn oil.

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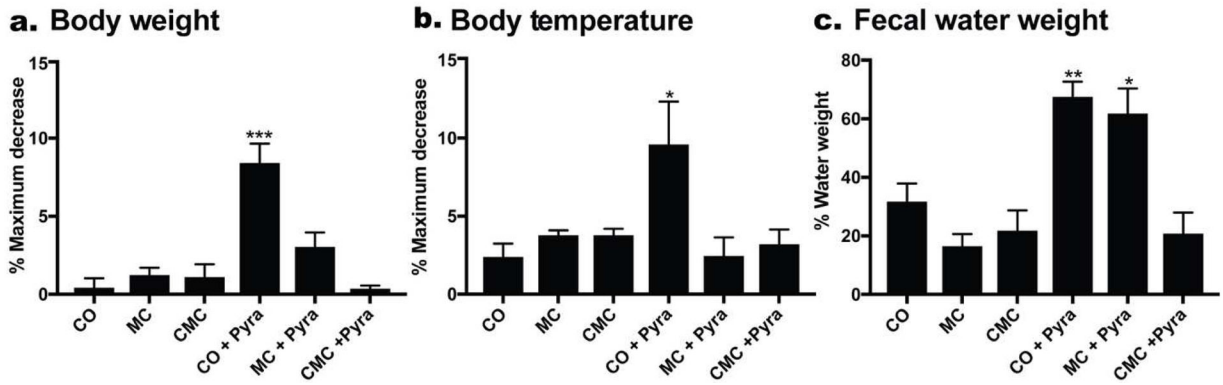

Fig. 2.

The effects of pyraclostrobin (400 mg/kg) dissolved in different vehicles on body weight **(a)**, core body temperature **(b)**, and fecal water weight **(c).** Symbols (n=5-8 male mice/vehicle/timepoint) represent mean ± SEM percentage of maximum possible decrease based on the comparison between pre-dosage baseline and measures taken at 3 hr and 24 hr after dosing for each mouse (see Experimental Procedures). Statistical comparisons are the result of one-way ANOVA followed by Bonferroni post hoc analysis * p<0.05, ** 0.01, *** 0.001 relative to CO control.

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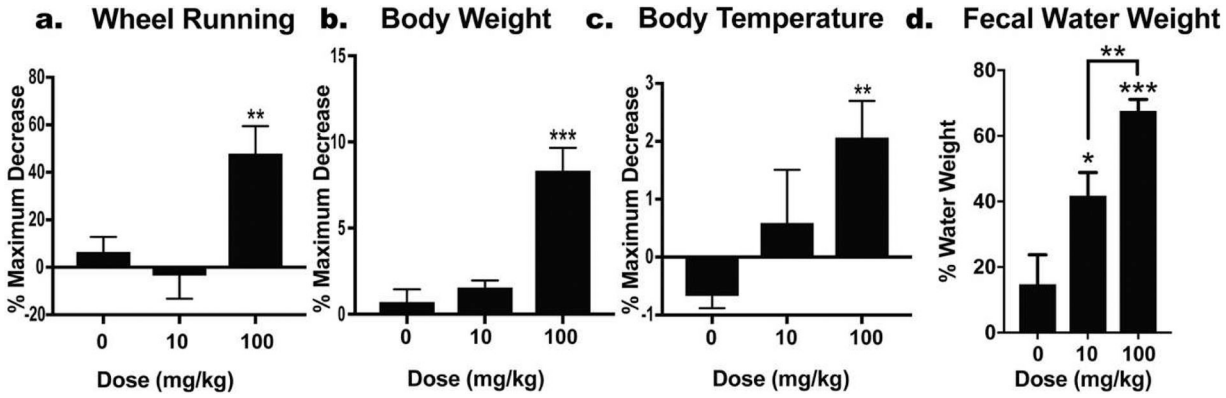

Fig. 3.

Dose-dependent effects of pyraclostrobin dissolved in corn oil on locomotor activity **(a),** body weight **(b)**, core body temperature **(c)**, and fecal water weight **(d).** Symbols (n=5-11 male mice/vehicle/timepoint) represent mean ± SEM percentage of maximum possible decrease based on the comparison between pre-dosage baseline and measures taken at 3 hr and 24 hr after dosing for each mouse (see Experimental Procedures). Statistical comparisons are the result of one-way ANOVA followed by Bonferroni post hoc analysis * p<0.05, ** 0.01, *** 0.001 relative to CO control.

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Table 1.

Amount of pyraclostrobin in dosing solutions. Vehicles are corn oil (CO), methyl cellulose (MC) or carboxymethyl cellulose (CMC). All samples were run three times (Replicate). Expected dose of pyraclostrobin was 0, 100, and 400 ng/ml.

Compound	Vehicle	Replicate	Expected Dose (ng/mL)	Observed Dose (ng/mL)	Replicate Dose Mean (ng/mL)
Corn Oil	CO	1	0	<MDL	
Corn Oil	CO	2	0	<MDL	
Corn Oil	CO	3	0	<MDL	**Undetected**
Pyraclostrobin	MC	1	100	7.06	
Pyraclostrobin	MC	2	100	<MDL	
Pyraclostrobin	MC	3	100	<MDL	**<7.06**
Pyraclostrobin	CMC	1	100	<MDL	
Pyraclostrobin	CMC	2	100	<MDL	
Pyraclostrobin	CMC	3	100	<MDL	**Undetected**
Pyraclostrobin	CO	1	100	104.21	
Pyraclostrobin	CO	2	100	126.71	
Pyraclostrobin	CO	1	100	113.20	**119.96**
Pyraclostrobin	MC	1	400	25.89	
Pyraclostrobin	MC	2	400	31.03	
Pyraclostrobin	MC	3	400	25.03	**28.03**
Pyraclostrobin	CMC	1	400	75.07	
Pyraclostrobin	CMC	2	400	75.80	
Pyraclostrobin	CMC	3	400	67.13	**71.47**
Pyraclostrobin	CO	1	400	374.64	
Pyraclostrobin	CO	2	400	397.10	
Pyraclostrobin	CO	3	400	425.09	**411.10**