Title: A neonicotinoid insecticide reduces fueling and delays migration in songbirds

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Abstract: Neonicotinoids are neurotoxic insecticides widely used as seed treatments, but little is known of their effects on migrating birds that forage in agricultural areas. We tracked the migratory movements of imidacloprid exposed songbirds at a landscape scale using a novel combination of experimental dosing and automated radio telemetry. Ingestion of field-realistic quantities of imidacloprid (1.2 or 3.9 mg·kg bm^{-1}) by white-crowned sparrows (*Zonotrichia leucophrys*) during migratory stopover caused a rapid reduction in food consumption, mass, and fat, and significantly affected probability of departure. High dose birds stayed a median of 3.5 days longer at the site of capture post-exposure compared to controls, likely to regain fuel stores or recover from intoxication. Migration delays can carry-over to affect survival and reproduction, thus these results provide a mechanistic link between sublethal pesticide exposure and adverse outcomes at the population level.

One Sentence Summary: Sparrows experimentally exposed to imidacloprid during spring migration exhibited anorexic behavior, reduced condition and extended stopover.

Main Text:

Birds are frequently exposed to pesticides and other environmental contaminants at migratory stopover sites, but studies that causally link contaminant exposure to migration ability are lacking. Migratory stopover is typically characterized by rapid food intake (hyperphagia) and assimilation of energy stores to prepare for sustained flight (*1*). Suppression of feeding and mass loss has been proposed as a sub-lethal mechanism through which certain neurotoxicants delay migration and reduce survival (*2*), while disruption of migratory orientation behavior has been identified as a sensitive endpoint of exposure to certain contaminants in songbirds (*3, 4*). Conditions experienced during migration have population-level consequences (*5*), and thus the

presence of contaminants at stopover sites could be contributing to the population declines
38 occurring in many migratory species.

There is increasing concern and controversy over the neonicotinoids, which are the most widely
40 used class of agricultural pesticides worldwide (*6*). Neonicotinoids have a neurotoxic mechanism
of action, binding to the nicotinic acetylcholine receptor (nAChR) causing overstimulation of the
42 nervous system (*7*). They bind more strongly to insect receptors than vertebrate receptors, and
were thought to pose a low risk for vertebrates (*7*). However, recent studies have demonstrated
44 that neonicotinoids can have significant negative effects on survival, condition and behavior in
birds (*8-10*). Birds can be exposed through multiple pathways, including contact with sprays,
46 ingestion of contaminated soil and water, as well as consumption of treated seeds. While
neonicotinoids have been on the market since the mid-1990s, researchers have only recently
48 started to focus on identifying field exposures in wildlife, and there is mounting evidence that
farmland birds worldwide are routinely exposed to neonicotinoids (*11-15*). Bird species that use
50 agricultural habitat for migration or breeding are exhibiting particularly precipitous declines (*16,
17*). In North America, 74% of farmland-dependent bird species declined from 1966-2013, many
52 of which are seed-eaters (*17*). Along with habitat loss and disturbance, the large-scale application
of agricultural pesticides, including the neonicotinoid seed treatments, has been associated with
54 these declines (*17, 18*), but detailed mechanistic studies are needed to establish a causal link.

Despite being an area of great scientific and conservation interest, the influence of
56 neonicotinoids on avian behavior, patterns of movement, and population-level effects remains
poorly understood. Birds are particularly susceptible to neonicotinoid exposure during spring
58 migration, which coincides with spring seeding for many treated agricultural crops in the
northern mid-latitudes (*19*). In a previous captive dosing study, we tested the effects of sub-lethal
60 imidacloprid exposure on migratory ability in a seed-eating songbird, the white-crowned sparrow
(*Zonotrichia leucophrys*), caught at stopover sites during their spring migration. Imidacloprid
62 caused strong acute effects, including rapid body mass (bm) loss and migratory disorientation
(*10*). To date, researchers have never been able to evaluate the effects of pesticide exposure on
64 free-living songbirds. New tracking technologies now permit insight into the consequences of
neonicotinoid exposure on songbirds at an ecologically relevant scale. In this study, we
66 combined controlled dosing of wild-caught birds (Fig. 1) and automated telemetry to track
fueling and migratory movements of individual white-crowned sparrows experimentally exposed
68 to very low sub-lethal doses of imidacloprid (3-10% of predicted median lethal dose) during a
northern stopover in Ontario, Canada. The selected doses were well within the range of
70 concentrations that a bird could realistically consume if they accidentally ingested a few treated
seeds (Table S1).

72 We found that even a single exposure to low doses of imidacloprid (1.2 mg·kg bm^{-1} or 3.9 mg·kg
bm^{-1}, n = 12 per treatment) caused negative effects on fueling and migration in sparrows.
74 Imidacloprid-induced mass loss has been reported in previous studies in birds (*8-10*), and we
observed a significant reduction in white-crowned sparrow body mass 6 hours post-dosing
76 (dose*time interaction: $F_{2,33}$ = 4.56, p = 0.018) in both the low and high dose (p = 0.005, average
mass loss = 3.0 %, and p < 0.0001, average mass loss = 5.9 %, respectively; Fig. 2a, Table S2).
78 Control birds subjected to the same procedures did not lose significant body mass (p = 0.156).
Nevertheless, it is possible that capture and handling may have been sufficient in dosed birds to
80 cause interactive effects with imidacloprid and amplify the magnitude of the dose response.
However, even after longer acclimation periods of ~2 weeks, our previous captive study in the

82 same species showed a significant negative effect on body mass after just one comparable dose of imidacloprid (4.0 mg·kg bm^{-1}·day^{-1}), with birds losing 6.5% of body mass within 24-hr after

84 the first dose and 17% of mass after three consecutive daily doses (*10*) (Table S3).

Fat is the essential fuel store in migrating birds (*20*), and body composition quantitative magnetic

86 resonance (QMR) scans confirmed that mass loss in imidacloprid exposed sparrows was driven by a corresponding loss of body fat (dose*time: $F_{2,33}$ = 3.90, p = 0.030), while lean mass loss

88 was similar across dose groups (dose*time: $F_{2,33}$ = 0.16, p = 0.855). Fat content in control birds did not change over time (p = 0.521), while fat significantly decreased after dosing in both the

90 low imidacloprid (p = 0.010; average fat loss = 9.3 %) and high imidacloprid (p < 0.0001; average fat loss = 17.1 %) birds (Fig. 2b, Table S2). Average lean mass decreased slightly (1.6

92 %) after dosing (time: $F_{1,33}$ = 10.82, p = 0.002; Fig. 2c, Table S2), but was not significantly different between dose groups (dose: $F_{2,33}$ = 2.52, p = 0.096).

94 The mass and fat loss in white-crowned sparrows appears to be associated in part with "anorexic" effects of imidacloprid. Food consumption during the 6-hour post-exposure period

96 was significantly reduced by imidacloprid ($F_{2,17}$ = 10.44, p = 0.001), with high dose birds on average eating 70 % less food compared to control birds on a body mass basis (Fig. 2d; p =

98 0.002). Low dose birds on average ate 8 % less food than controls, although this decrease was not significant (p = 0.898). Reduced food intake was not related to aversion of seeds, as exposure

100 was through oral gavage and all food provided was untreated. Other avian imidacloprid dosing studies have similarly observed suppression of feeding behavior (*9, 21*). It is not clear if appetite

102 suppression alone is the cause for reduced body mass in exposed birds; other interactive effects of imidacloprid may have altered nutrient assimilation efficiency or caused general toxicity that

104 could exacerbate mass loss. The anorexic properties of nicotine have been associated with central cholinergic-linked metabolic processes (*22*), which provides some insight into the possible

106 mechanistic links between nAChR agonists, such as nicotine and neonicotinoids, and appetite suppression.

108 We found strong evidence that imidacloprid exposure significantly extended stopover duration (Fig. 3, Tables S4-S5). The top model that included dose, plus intrinsic (fat, time in captivity)

110 and extrinsic (weather) covariates, had the strongest support and significantly predicted the likelihood of departure (χ^2 = 41.4, df = 5, p < 0.001, n events = 33), while support for the second

112 model without dose was weak (> 7 ΔAICc)(Table S4). For all 33 birds detected at departure, the median stopover duration was 2 days (range 0 to 9 days). The longest stopover durations were

114 observed in dosed birds (control median = 0.5 days, range = 0 to 4 days; low dose median = 3 days, range = 0 to 8 days; high dose median = 4 days, range = 0 to 9 days). High dose birds were

116 only 11.7% as likely to depart under similar conditions as control birds (Hazard Ratio [HR] = 0.117, 95% CI = 0.024 – 0.453; Table S5). Low dose birds were 87.2% as likely to depart as

118 control birds, however 95% CI for probability of departure closely overlapped with controls (Fig. 3, HR = 0.872, 95% CI = 0.272 – 2.754). We also found that birds that were held for longer

120 before being screened into the experiment tended to have shorter stopover durations after release (HR = 2.399, 95% CI = 1.261 – 5.129), and a higher weather index (higher temperature and

122 tailwinds) was associated with a greater probability of departure and shorter stopover durations (HR = 2.356, 95% CI = 1.514 – 3.995). Pre-dosing fat was not a good predictor of stopover

124 duration, as 95% confidence intervals overlapped one (HR = 1.261, 95% CI = 0.947 – 1.665).

We hypothesize that the extended stopover in imidacloprid exposed birds was related to the

126 reduced fuel loads and suppression of feeding and fueling ability. Previous studies have found

128 that *zugunruhe* (migratory restlessness) and the timing of migratory flight are tightly tied with fat accumulation rate (*23*), and the amount of fuel lost is a better predictor of stopover duration than absolute fuel stores (*24*). Birds that lose more fuel stores during fasting periods tend to have

130 lower *zugunruhe* once they start refueling, and the motivation to depart is suppressed until sufficient fuel stores for the next flight have been replenished (*24*). It is probable that birds in the

132 high dose imidacloprid exposed groups, which lost more fat, would need more time to regain fuel stores, resulting in the longer observed stopovers.

134 While captive experiments of white-crowned sparrows exposed to imidacloprid showed disrupted migratory orientation (*10*), we did not find any effects on flight path or orientation in

136 free-living sparrows. There was no statistical difference between the mean overall orientation of any of the dose groups ($F_{2,27} = 1.882$, $p = 0.172$) (Fig. 4), and the mean overall orientation

138 bearing was significantly oriented in a northeast direction (44.1°) for all dose groups, including controls (Rayleigh Test: $Z_{30} = 28.1$, $p < 0.0001$). Similarly, the departure bearings were

140 consistent across treatments ($F_{2,27} = 1.17$, $p = 0.326$), and the mean departure bearing was slightly more north at 32.6° (Rayleigh Test: $Z_{30} = 27.0$, $p < 0.0001$). In addition, the average

142 sustained migratory flight speed was similar across treatment groups (20.1 ± 1.6 m·s^{-1}, $F_{2,23} = 0.29$, $p = 0.753$).

144 The lack of effects on orientation and speed in our field study suggest that free-living birds avoid migratory flight while recovering from intoxication. Imidacloprid nAChR binding is reversible

146 (*7, 25*), and birds are able to recover from non-lethal exposures. In the previous captive study, birds tested in orientation funnels regained both body mass and orientation ability within two

148 weeks following the completion of dosing (*10*). The extended stopover we observed in birds exposed to a single high dose likely served as a self-imposed recovery period to regain fuel

150 stores, or recover from neurotoxic effects.

151 Extended stopovers while intoxicated and in reduced body condition could lower survival by

152 increasing susceptibility to predation or inclement weather. Imidacloprid exposure could also reduce fitness through the sub-lethal consequences of longer stopovers. There are selective

154 pressures for birds to minimize the time spent migrating (*26*). Birds that are delayed during migration and arrive later at breeding grounds have been reported to obtain poorer quality

156 territories, breed later, and produce fewer offspring in worse condition than early arrivals, reducing the probability of their offspring recruitment to the population (*27, 28*). The seed-

158 treatment application rates of common neonicotinoids (e.g. imidacloprid) for several crops are at concentrations where only few treated seeds (< 5) need to be consumed to reach the level of

160 concern for acute lethality in small and medium sized birds (*29*). Treated seed avoidance has been documented, but is a learned response rather than innate sensory aversion to neonicotinoid

162 seed coatings, so offers little protection to naïve birds (*30, 31*). Birds will consume spilled or near surface seeds after planting as a food source (*11, 15*), and there are reports of free-ranging

164 birds consuming enough treated seeds to reach lethal concentrations (*14, 32*). The widespread use of neonicotinoids along migratory routes in the United States and southern Canada means

166 that individuals may experience repeated exposure at successive stopover sites, resulting in cumulative delays which would amplify these negative fitness consequences.

168 This is the first time researchers have been able to track the fate of free-living pesticide-exposed birds over ecologically relevant spatial scales. Through the use of controlled dosing and

170 automated telemetry, we were able to discern that improper flight paths caused by disorientation are likely not the main concern for imidacloprid exposure, as birds appear to avoid flight while

172 intoxicated or regaining body fat. Instead, the neonicotinoids act as anorexic agents causing mass loss during a critical life stage that is typically characterized by hyperphagia and rapid fat
174 accumulation. In addition to directly decreasing survival when consumed at acutely toxic concentrations (*14, 32*), ingestion of minute sub-lethal quantities of imidacloprid during the
176 critical migratory stopover period causes delays that could also reduce future fitness. The sub-lethal effects of imidacloprid on food consumption, body condition, and stopover duration have
178 clear links with survival and reproduction, and are predicted to negatively affect populations of migratory birds that commonly use agricultural habitats for refueling.

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References:

182 1. F. Bairlein, E. Gwinner, Nutritional mechanisms and temporal control of migratory energy accumulation in birds. *Annu. Rev. Nutr.* **14**, 187-215 (1994).

184 2. J. E. Elliott, C. Bishop, in *Environmental Contaminants in Biota,* W. N. Beyer, J. P. Meador, Eds. (CRC Press, 2011), pp. 447-475.

186 3. L. Flahr, N. L. Michel, A. Zahara, P. D. Jones, C. A. Morrissey, Developmental exposure to Aroclor 1254 alters migratory behaviour in juvenile European starlings (*Sturnus*
188 *vulgaris*). *Environmental Science & Technology*, (2015).

    4. N. B. Vyas, E. F. Hill, J. R. Sauer, W. J. Kuenzel, Acephate affects migratory orientation
190 of the white-throated sparrow (*Zonotrichia albicollis*). *Environ. Toxicol. Chem.* **14**, 1961-1965 (1995).

192 5. I. Newton, Can conditions experienced during migration limit the population levels of birds? *J Ornithol* **147**, 146-166 (2006).

194 6. N. Simon-Delso *et al.*, Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental Science and Pollution Research* **22**, 5-34
196 (2015).

    7. M. Tomizawa, J. E. Casida, Selective toxicity of neonicotinoids attributable to specificity
198 of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol.* **48**, 339-364 (2003).

    8. A. Lopez-Antia, M. E. Ortiz-Santaliestra, F. Mougeot, R. Mateo, Imidacloprid-treated
200 seed ingestion has lethal effect on adult partridges and reduces both breeding investment and offspring immunity. *Environ. Res.* **136**, 97-107 (2015).

202 9. A. Lopez-Antia, M. E. Ortiz-Santaliestra, F. o. Mougeot, R. Mateo, Experimental exposure of red-legged partridges (*Alectoris rufa*) to seeds coated with imidacloprid,
204 thiram and difenoconazole. *Ecotoxicology* **22**, 125-138 (2013).

    10. M. L. Eng, B. J. M. Stutchbury, C. A. Morrissey, Imidacloprid and chlorpyrifos
206 insecticides impair migratory ability in a seed-eating songbird. *Sci. Rep.* **7**, 15176 (2017).

    11. A. Lopez-Antia, J. Feliu, P. R. Camarero, M. E. Ortiz-Santaliestra, R. Mateo, Risk
208 assessment of pesticide seed treatment for farmland birds using refined field data. *J. Appl. Ecol.* **53**, 1373-1381 (2016).

210   12.   C. Hao, M. L. Eng, F. Sun, C. A. Morrissey, Part-per-trillion LC-MS/MS determination
              of neonicotinoids in small volumes of songbird plasma. *Sci. Total Environ.* **644**, 1080-
212   1087 (2018).

       13.   S. Humann-Guilleminot *et al.*, A large-scale survey of house sparrows feathers reveals
214   ubiquitous presence of neonicotinoids in farmlands. *Sci. Total Environ.* **660**, 1091-1097
              (2019).

216   14.   C. J. Botha, E. C. Du Plessis, H. Coetser, M. Rosemann, Analytical confirmation of
              imidacloprid poisoning in granivorous Cape spurfowl (*Pternistis capensis*). *J S Afr Vet*
218   *Assoc* **89**, 1-5 (2018).

       15.   C. L. Roy, P. L. Coy, D. Chen, J. Ponder, M. Jankowski, Multi-scale availability of
220   neonicotinoid-treated seed for wildlife in an agricultural landscape during spring
              planting. *Sci. Total Environ.* **682**, 271-281 (2019).

222   16.   P. F. Donald, F. J. Sanderson, I. J. Burfield, F. P. J. van Bommel, Further evidence of
              continent-wide impacts of agricultural intensification on European farmland birds, 1990-
224   2000. *Agric., Ecosyst. Environ.* **116**, 189-196 (2006).

       17.   R. L. Stanton, C. A. Morrissey, R. G. Clark, Analysis of trends and agricultural drivers of
226   farmland bird declines in North America: A review. *Agric., Ecosyst. Environ.* **254**, 244-
              254 (2018).

228   18.   C. A. Hallmann, R. P. B. Foppen, C. A. M. van Turnhout, H. de Kroon, E. Jongejans,
              Declines in insectivorous birds are associated with high neonicotinoid concentrations.
230   *Nature* **511**, 341-343 (2014).

       19.   W. J. Sacks, D. Deryng, J. A. Foley, N. Ramankutty, Crop planting dates: an analysis of
232   global patterns. *Global Ecol. Biogeogr.* **19**, 607-620 (2010).

       20.   C. G. Guglielmo, Obese super athletes: fat-fueled migration in birds and bats. *The*
234   *Journal of Experimental Biology* **221**, jeb165753 (2018).

       21.   L. M. Addy-Orduna, J. C. Brodeur, R. Mateo, Oral acute toxicity of imidacloprid,
236   thiamethoxam and clothianidin in eared doves: A contribution for the risk assessment of
              neonicotinoids in birds. *Sci. Total Environ.* **650**, 1216-1223 (2019).

238   22.   Y. H. Jo, D. A. Talmage, L. W. Role, Nicotinic receptor-mediated effects on appetite and
              food intake. *J. Neurobiol.* **53**, 618-632 (2002).

240   23.   Å. Lindström, in *Avian Migration,* P. Berthold, E. Gwinner, E. Sonnenschein, Eds.
              (Springer Berlin Heidelberg, Berlin, Heidelberg, 2003), pp. 307-320.

242   24.   C. Eikenaar, F. Bairlein, Food availability and fuel loss predict Zugunruhe. *J Ornithol*
              155, 65-70 (2014).

244   25.   M. Y. Liu, J. E. Casida, High affinity binding of [^{3}H]imidacloprid in the insect
              acetylcholine receptor. *Pestic. Biochem. Physiol.* **46**, 40-46 (1993).

246   26.   T. Alerstam, Optimal bird migration revisited. *J Ornithol* **152**, 5-23 (2011).

       27.   H. Kokko, Competition for early arrival in migratory birds. *J. Anim. Ecol.* **68**, 940-950
248   (1999).

28. R. J. Smith, F. R. Moore, Arrival timing and seasonal reproductive performance in a long-distance migratory landbird. *Behav. Ecol. Sociobiol.* **57**, 231-239 (2005).

29. Health Canada, Imidacloprid: Proposed Re-evaluation Decision. *Pest Management Regulatory Agency* **PRVD2016-20** (2016).

30. A. Lopez-Antia, M. E. Ortiz-Santaliestra, R. Mateo, Experimental approaches to test pesticide-treated seed avoidance by birds under a simulated diversification of food sources. *Sci. Total Environ.* **496**, 179-187 (2014).

31. M. L. Avery, D. G. Decker, D. L. Fischer, T. R. Stafford, Responses of captive blackbirds to a new insecticidal seed treatment. *The Journal of Wildlife Management* **57**, 652-656 (1993).

32. F. Millot *et al.*, Field evidence of bird poisonings by imidacloprid-treated seeds: a review of incidents reported by the French SAGIR network from 1995 to 2014. *Environmental Science and Pollution Research* **24**, 5469-5485 (2017).

33. G. Chilton, M. C. Baker, C. D. Barrentine, M. A. Cunningham, in *The Birds of North America,* A. F. Poole, F. B. Gill, Eds. (Cornell Lab of Ornithology, Ithaca, NY, USA., 1995).

34. L. M. Flahr, N. L. Michel, A. R. D. Zahara, P. D. Jones, C. A. Morrissey, Developmental exposure to Aroclor 1254 alters migratory behavior in juvenile European starlings (*Sturnus vulgaris*). *Environ. Sci. Technol.* **49**, 6274-6283 (2015).

35. S. T. Emlen, J. T. Emlen, A technique for recording migratory orientation of captive birds. *The Auk* **83**, 361-367 (1966).

36. R. Muheim, J. B. Phillips, S. Akesson, Polarized light cues underlie compass calibration in migratory songbirds. *Science* **313**, 837-839 (2006).

37. R. Muheim, I. Henshaw, S. Sjöberg, M. E. Deutschlander, BirdOriTrack: a new video-tracking program for orientation research with migratory birds. *J. Field Ornithol.* **85**, 91-105 (2014).

38. C. G. Guglielmo, L. P. McGuire, A. R. Gerson, C. L. Seewagen, Simple, rapid, and non-invasive measurement of fat, lean, and total water masses of live birds using quantitative magnetic resonance. *J Ornithol* **152**, 75 (2011).

39. L. V. Kennedy, Y. E. Morbey, S. A. Mackenzie, P. D. Taylor, C. G. Guglielmo, A field test of the effects of body composition analysis by quantitative magnetic resonance on songbird stopover behaviour. *J Ornithol* **158**, 593-601 (2017).

40. P. D. Taylor *et al.*, The Motus Wildlife Tracking System: a collaborative research network to enhance the understanding of wildlife movement. *Avian Conserv Ecol* **12**, 8 (2017).

41. A. M. Mills, B. G. Thurber, S. A. Mackenzie, P. D. Taylor, Passerines use nocturnal flight for landscape-scale movements during migration stopover. *Condor.* **113**, (2011).

42. M. Kanamitsu *et al.*, NCEP-DOE AMIP-II Reanalysis (R-2). *Bulletin of the American Meteorological Society* **83**, 1631-1644 (2002).

288 43.   M. U. Kemp, E. Emiel van Loon, J. Shamoun-Baranes, W. Bouten, RNCEP: global
            weather and climate data at your fingertips. *Methods in Ecology and Evolution* **3**, 65-70
290   (2012).

     44.   G. W. Mitchell, B. K. Woodworth, P. D. Taylor, D. R. Norris, Automated telemetry
            reveals age specific differences in flight duration and speed are driven by wind conditions
292   in a migratory songbird. *Movement Ecology* **3**, 19 (2015).

294 45.   M. U. Kemp *et al.*, Quantifying flow-assistance and implications for movement research.
            J. Theor. Biol. **308**, 56-67 (2012).

296 46.   R Core Team, R: A language and environment for statistical computing. R Foundation
            for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. (2018).

298 47.   SAS Institute. (SAS Institute Inc., Cary, NC, USA., 2012).

     48.   P. D. Allison, *Survival analysis using SAS: a practical guide*. (SAS Institute, ed. 2nd,
300   2010).

     49.   M. Ramenofsky, R. Agatsuma, T. Ramfar, Environmental conditions affect the behavior
302   of captive, migratory white-crowned sparrows. *The Condor* **110**, 658-671 (2008).

     50.   P. Mineau, C. Palmer, The impact of the nation's most widely used insecticides on birds.
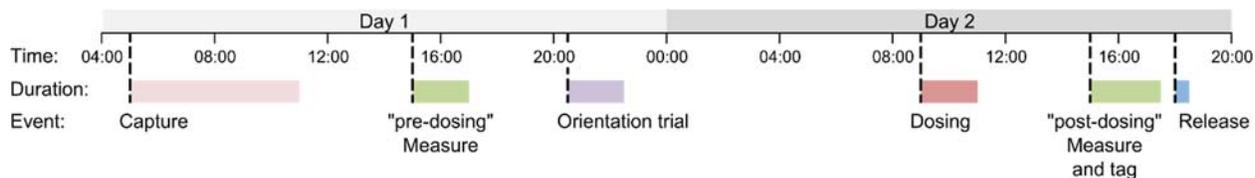304   *American Bird Conservancy*, (2013).

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**Supplementary Materials:**

324 Materials and Methods

Tables S1-S5

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Fig. 1. Experimental timeline for each cohort of white-crowned sparrows captured on migration stopover. The same body measurements were taken ~ 24 hours apart to compare "pre-dosing" and "post-dosing" condition. Nocturnal orientation trials tested for baseline migratory activity and orientation, and only birds with fat filling ≥ ½ the furcular hollow and exhibiting migratory restlessness were screened into the dosing study. Birds were orally dosed the following morning, measured and nanotagged ~6-hours post-dosing, and then released 2-hours before sunset. Post-release tracking was accomplished remotely via the Motus Wildlife Tracking System.

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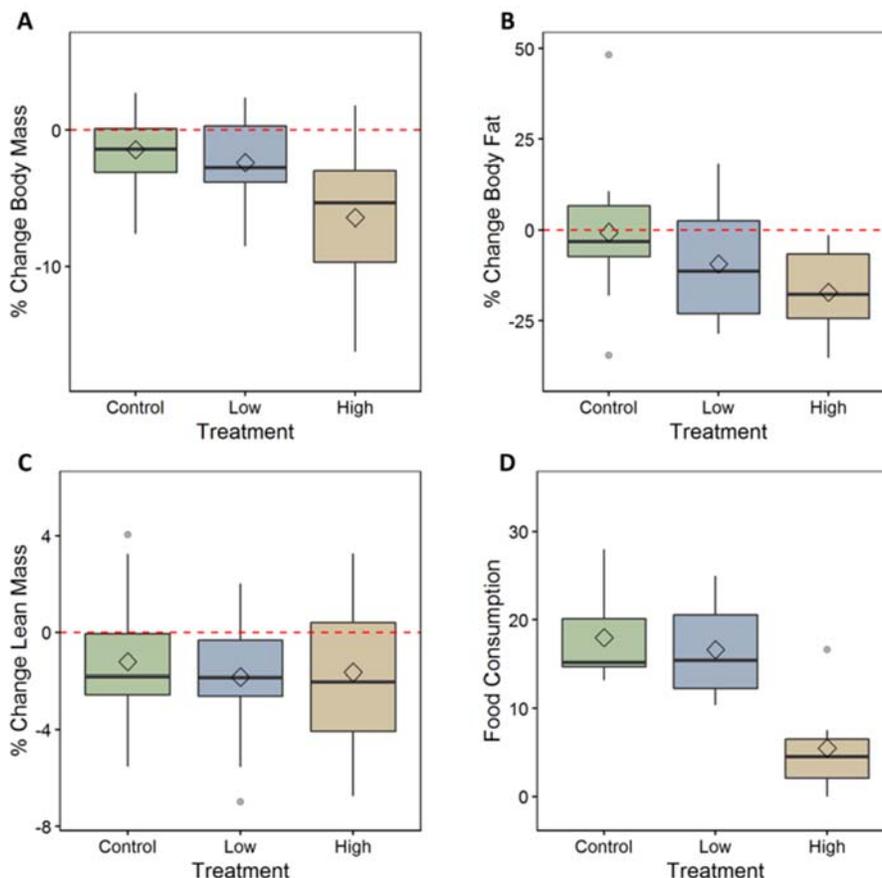
Fig. 2. Effects of a single oral dose of imidacloprid on body condition and food consumption in migrating white-crowned sparrows. Percent change between pre-dosing and 6 hours post-dosing for (A) body mass, (B) fat, and (C) lean mass in white-crown sparrows. (D) Food consumption (g food·kg body mass^{-1}·hour^{-1}) over the 6-hour post-dosing period. Control = vehicle sunflower oil, low = 1.2 mg·kg bm^{-1}), high = 3.9 mg·kg bm^{-1}. Boxes indicate interquartile range, middle lines indicate median, diamonds indicate the mean, whiskers show the minimum and maximum values within 1.5x the inter-quartile range, and dots represent outliers (>1.5x inter-quartile range from box). n = 12 birds per dose group, food consumption based on averages per cage (1 to 3 birds/cage) within the same treatment.

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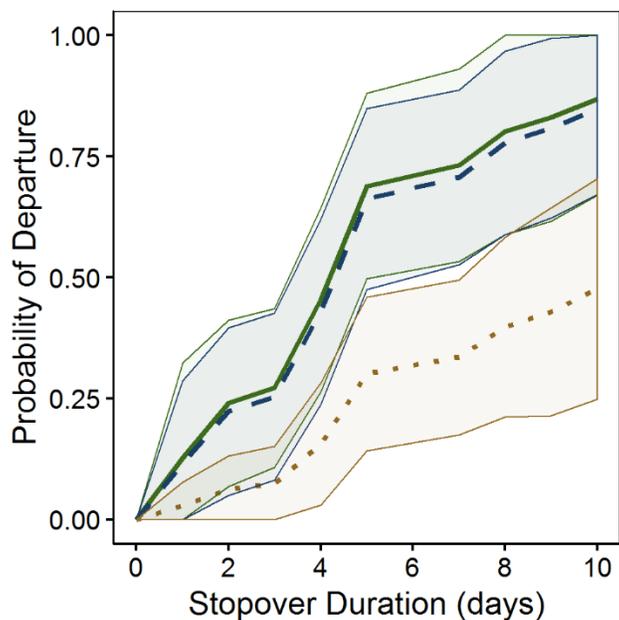
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Fig. 3. Effect of a single oral imidacloprid exposure on stopover duration in migrating white-crowned sparrows. Lines indicate predicted probability of departure from the stopover site for each dose group over time (solid green = control, dashed blue = low, dotted brown = high; n = 12 control, 10 low, 11 high). Estimates are adjusted for weather conditions, time in captivity, and pre-dosing fat loads. Shaded area represents 95% confidence intervals which overlap for the control and low dose treatments. The probability of departure for high dose (3.9 mg·kg bm^{-1}) birds was 8.5 times lower than controls (sunflower oil vehicle).

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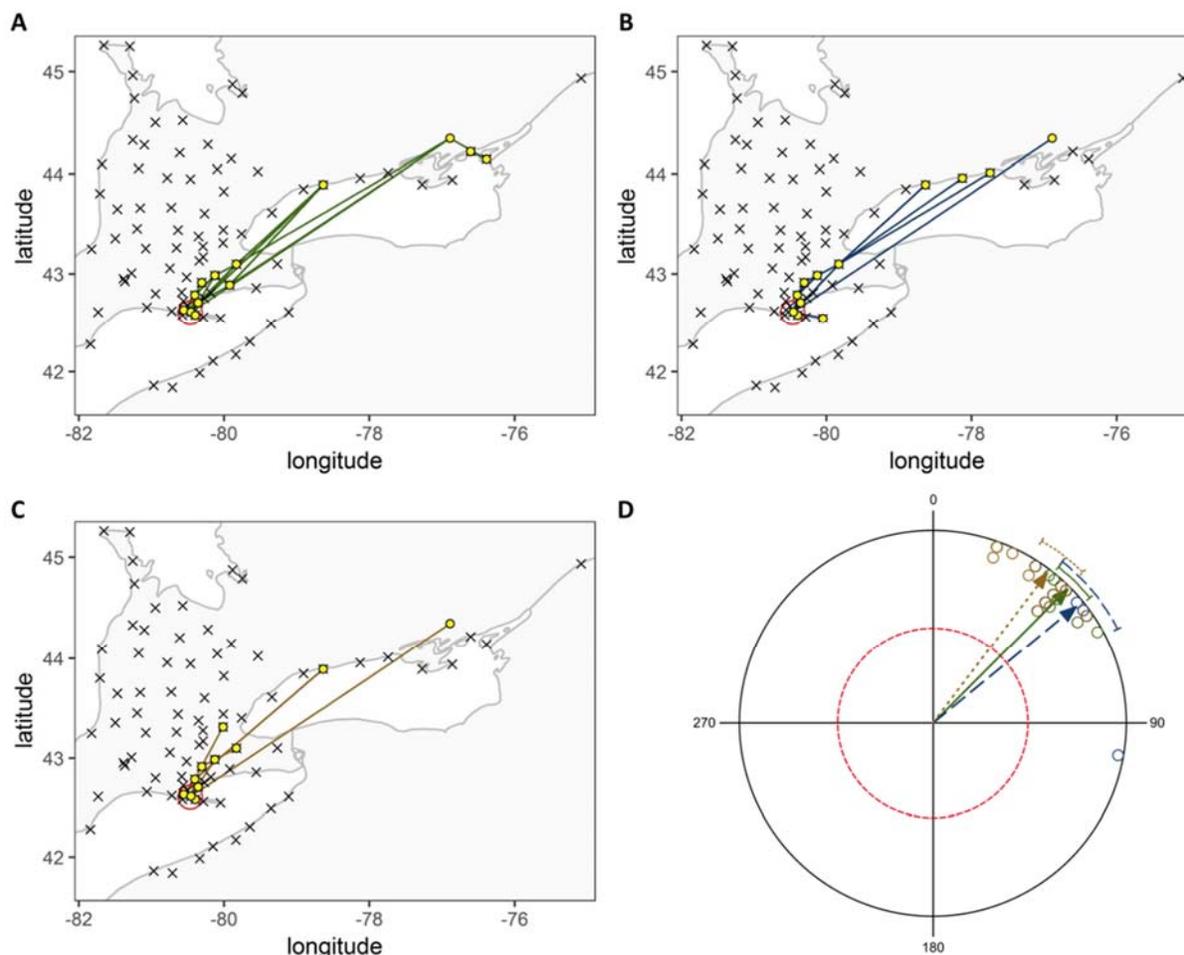
Fig. 4. Migratory flight paths and overall bearings for 30/33 detected white-crowned sparrows tracked in an automated telemetry array in the southern Ontario, Canada. Flight paths of control (**A**), low imidacloprid (1.2 mg·kg bm^{-1}) (**B**), and high imidacloprid (3.9 mg·kg bm^{-1}) (**C**) dosed birds. Red circle indicates capture and release site near Long Point, Ontario, yellow circles indicate tag detections, lines are drawn between sites with consecutive detections and do not represent actual flight paths, × indicates locations of active radio telemetry receivers during the lifespan of the nanotags. (**D**) Overall bearing of detection paths. Solid green = control, dashed blue = low, dotted brown = high. Open circles represent the bearing of individual birds and arrows represent mean orientation of each treatment group, the length of the arrows indicates how closely individuals are clustered around the mean, dashed red line indicates the critical values for Rayleigh's uniformity test at $\alpha = 0.05$ (vectors that pass this critical value are significant), and the outer arc represents the 95% confidence interval for each significant vector.

Science

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Supplementary Materials for

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Materials and Methods

Study location and species

White-crowned sparrows were selected as a representative seed-eating migratory songbird. In the spring of 2017, we captured migrating eastern white-crowned sparrows (*Z. l. leucophrys*) in mist nets or sparrow traps on stopover at Long Point Bird Observatory, located in Southern Ontario, Canada (42.5829° N, 80.3985° W). Eastern white-crowned sparrows overwinter from central Texas to the lower Ohio River Valley, and breed in boreal shrub habitat in north eastern Canada (*33*). Birds were captured starting at 30 minutes before sunrise until 5.5 hours after sunrise, and each individual was banded with a standard US Fish and Wildlife Service aluminum band. Between capture and release, birds were held in cages (66 cm L x 46 cm W x 50 cm H) on site in an animal housing room within a mobile research laboratory, with 1 to 3 birds per cage, and provided with water and a mixture of millet and black oil sunflower seeds *ad libitum*. Research protocols were in compliance with the Canadian Council on Animal Care guidelines and approved by the University of Saskatchewan Animal Care Committee (AUP 20110043), and conducted under Canadian Wildlife Service Scientific Permit SC00008. Details of dosing, behavioural trials and telemetry are below, and the timeline for experimental procedures is illustrated in Fig. 1.

Pre-dosing activity screening

A baseline measure of migratory activity and orientation was taken using nocturnal orientation (Emlen) funnel trials using previously described methods (*10, 34, 35*). To minimize variation and increase likelihood that birds were in a migratory state, only birds with fat filling at least half the furculum as well as fat present in the wing pits and abdominal regions were tested in orientation funnels. Funnel trials were conducted in a field located 6 km from capture site and approximately 1 km from a Motus tower (BSC HQ). Birds were brought to the field at least 30 minutes before sunset, and held in a standard cage that was elevated 40 cm off of the ground to allow a full unobstructed view of the sunset aiding calibration of the magnetic compass (*36*). Immediately after sunset was complete, birds were transferred to individual Emlen funnels. Behaviour was video recorded starting at least 15 minutes before civil twilight, until one hour after civil twilight. Immediately after completion of trials, BirdOriTrack software (*37*) was used to collect activity and orientation data from recorded video. Migratory activity was measured as cumulative distance moved via outward hops in the funnels over 30 sec intervals. Birds that had

activity scores > 100 and a significant northward (270° to 90°, adjusted for magnetic declination) orientation were considered to be in a state of *zugunruhe* (migratory restlessness) and were screened into the dosing experiment. Birds that were not active were held overnight and re-tested the following evening.

Imidacloprid dosing and blood sampling

The following morning (09:00 to 11:00) birds that were screened into the study were weighed and then dosed. Birds were randomly assigned to treatment groups, and dosing was through oral gavage using 20G curved stainless steel tube feeders at a volume of 10 ml per kg body mass (bm), with either the low dose (1.2 mg imidacloprid·kg bm^{-1}; n = 12), high dose (3.9 mg imidacloprid·kg bm^{-1}; n = 12), or vehicle control (sunflower oil; n = 12). Doses for this field study were selected to be sub-lethal based on prior captive studies (*10*) for this species. Based on typical white-crowned sparrow food consumption rates and current US EPA imidacloprid application rates, the high dose was the equivalent to consuming <1.5% imidacloprid treated seeds in the daily sparrow diet, and the low dose is equivalent to <0.5% of the diet being treated seeds (Table S1). Dosing solution composition and analysis has been previously described (*12*). Briefly, solutions were composed of technical grade imidacloprid (Sigma Aldrich 37894) mixed with food-grade organic sunflower oil (Compliments brand, Sobeys Canada) as a vehicle. Nominal concentrations were 0 mg imidacloprid·ml^{-1} (control), 0.10 mg imidacloprid·ml^{-1} (low), and 0.41 mg imidacloprid·ml^{-1} (high), based on 2.5 % or 10 % of the median lethal dose (LD50) for house sparrows (*Passer domesticus*), which is 41 mg·kg bm^{-1}. Dosing solution concentrations of imidacloprid were confirmed by LC-MS/MS analyses at the National Hydrology Research Centre, Environment and Climate Change Canada, Saskatoon, SK, and measured concentrations of imidacloprid were 0.12 mg·ml^{-1} (low) and 0.39 mg·ml^{-1} (high). Vehicle control oil and all blanks had no detectable levels of imidacloprid. Blood-samples were taken at capture and ~6 hours after dosing for neonicotinoid analysis, and circulating imidacloprid plasma concentrations resulting from controlled oral dosing have previously been described (*12*). Imidacloprid concentrations were significantly elevated in blood plasma samples in a dose-dependent manner (control geometric mean (GM) = 28.1 ng·ml^{-1} [range ND to 80.1 pg·ml^{-1}], low GM = 234.2 pg·ml^{-1} [range 100 to 3210 pg·ml^{-1}], high GM = 5558.2 pg·ml^{-1} [range 840 to 35,100 pg·ml^{-1}]) six hours after a single oral dose (0, 1.2 or 3.9 mg imidacloprid·kg bm^{-1}), as previously reported (*12*).

Body composition

Pre- and post- dosing lean mass and fat were measured using a quantitative magnetic resonance (QMR) body composition analyzer (Echo MRI-B, Echo-Medical Systems, Houston, TX, USA) (*38*) at approximately the same time of day (between 15:00 to 17:30) on two consecutive days (Fig 1), when body mass (g) was also measured. On average, "post-dosing" QMR measures were taken 6.1 ± 0.1 hours after dosing. Each sparrow was placed in a 33 mm diameter clear plastic tube to gently restrain, then measurements were made in duplicate using the two accumulation and 'small bird' settings of the QMR (total scan duration 2 min). A 3g oil standard was run daily, and the average reading was 3.00 ± 0.03 g (mean $\pm$ SE). Fat and lean mass values were corrected using calibration equations from Guglielmo et al. (*38*). The lean mass primarily represents muscles and organs, and fat mass primarily represents energy stored as fat as well as small amounts of lipids in membranes. Previous studies have confirmed the magnetic fields associated with QMR do not affect the stopover duration or orientation of migratory birds (*39*), and all birds had a full view of the sunset on the evenings of orientation trials and releases.

Food consumption

Cages were cleaned immediately prior to dosing, and food provided in shallow trays was weighed. Birds were placed in cages with up to two other individuals from the same treatment group due to space constraints and to maintain social interactions. At approximately 6 hours post-dosing birds were removed from cages, and total food remaining was weighed. Spilled food was included by collecting off of the cage floor, and feces were removed from the food dish prior to weighing. Food consumption was calculated for each cage by dividing the total food consumption by the total body mass of all birds in the cage, on a per hour basis (g food·kg bm^{-1}·hour^{-1}) (n cages = 7 control, 6 low, 7 high).

Migratory direction and speed

An hour before release, birds were individually tagged by gluing between the scapulars a coded nanotag (lightweight VHF transmitters; Lotek NTQB-2 0.35 g, 5.3 second burst, 166.380 MHz Motus frequency, tag and glue <2% total body mass), then transported to the field where funnel trials took place. At least 2 hours prior to sunset (18:00 to 18:30, with the exception of the first cohort of birds [n = 3] that were released after sunset), birds were soft released from the transport cage into the field by opening the cage door and allowing birds to fly out. Migratory movements were tracked using the Motus Wildlife Tracking System (Motus), an automated radio

telemetry network composed of receiver towers that can pick up radio signals from tagged animals when they fly within the range of detection (~15 km) (*40*) (Fig. 4). In southern Ontario during the period of this study, there were 93 operating towers providing broad coverage of the region. There were three towers within the detection range of the release site that detected tagged birds (BSC HQ [1.3 km], Old Cut [6.4 km], and Walsingham Super Tower [7.9 km]). Birds were released in six cohorts over an eight-day period. To minimize bias introduced from seasonal effects and individual variation, treatments were distributed approximately evenly across cohorts, and only birds with higher fat scores were included in the experiment. Tailwinds ($F_{2,32} = 0.11$, p $= 0.896$) and temperature ($F_{2,32} = 0.51$, p $= 0.606$) at release, and pre-dosing fat ($F_{2,33} = 0.02$, p $= 0.976$) were similar for all treatments.

Departure flights were confirmed for 33 out of 36 tagged birds using automated telemetry (n $= 12$ control, 10 low, 11 high). One of the low dose birds that was not detected was due to the tag failure at release (confirmed by manual telemetry). Flight tracks of each individual bird were screened by visualizing the signal strength in relation to time at each tower location to identify and remove false or ambiguous detections. To determine stopover duration for each bird, time of final departure was identified using graphs of signal strength over time from the tower adjacent to the release site ("BSC HQ"). Departure signals coincided with the last day the bird was manually detected at the release site, and were characterized by a series of detections that progressively increased and then decreased in signal strength, corresponding with when the bird flew towards then away from the tower (*41*). Stopover duration was calculated as the number of days between release and first detection of departing migratory flight.

We were able to calculate overall and departure bearings from 30/33 tagged birds (n $= 11$ control, 10 low, 9 high) that were detected flying by a Motus telemetry tower further than the detection range (15km) from the release site. The overall orientation of each bird was calculated as the bearing between the release site and the last location detected, and the departure bearing was calculated as the bearing between the release site and the next location detected. To estimate the flight speed of individual birds, the great-circle distance (metres) between consecutive sites was calculated using the haversine formula, and divided by the time in flight (seconds) between the same sites. The time stamp of maximum signal strength for each tower was considered the time of flyby. The signal strength patterns for detections at all tower detections other than those within range of the release site were characteristic of flybys rather than stopovers. To identify

sustained migratory flight speeds, we filtered out unrealistic flight times (> 100 m$^-\cdot$s^{-1}) that were the result of simultaneous detections at more than one tower, as well as the first detection after a stopover that could be identified by flight durations > 0.5 days between towers. Six flybys (out of 112) were filtered out because they were > 0.5 days from the last detection. We were able to calculate tower to tower flight speeds for 26 birds (n = 10 control, 9 low, 7 high).

Weather data

   Weather data was retrieved from the National Center for Environmental Prediction (NCEP)/ Department of Energy (DOE) Reanalysis 2 data set (http://www.esrl.noaa.gov/psd/data/gridded/data.ncep.reanalysis2.html) (42) using the "NCEP.interp" function in the RNCEP package in R (43). Wind and temperature at the release site was interpolated for each possible day of departure at the average time of departure (23:07 EDT). The east/west (U) and north/south (V) wind speeds in m$\cdot$s^{-1} were interpolated at the 925 mbar pressure level (~750-850 m altitude), which is realistic for migrating sparrows (44). Air temperature at 2 m altitude with reference to the surface was interpolated using the Reanalysis data set. Estimated tailwinds were calculated as $y\cdot\cos\theta$, where y is wind flow speed, and θ is (wind direction – preferred direction of movement) (45). To calculate the tailwind on each possible day of departure for departure decision analysis, the preferred direction of movement was set as the average overall flight bearing determined from tracking (44.1°). For comparisons of tailwind at release, the preferred direction was set as the flight bearing of each bird or the average bearing for birds that were only detected at departure.

Statistical analysis

   Telemetry filtering and migratory movement analysis were completed using R version 3.5.1 (46), body condition and stopover duration analysis was completed in SAS 9.4 (47), and circular orientation statistics were completed in Oriana version 4.0 (Kovach Computing Services). Assumptions of normality and homogeneity of variance were tested for using Shapiro-Wilk and Levene's tests, and by examining Q-Q plots, and where necessary data were log transformed to meet assumptions. Differences between pre-dosing and post-dosing total body mass, fat mass, and lean mass were assessed using linear mixed-effects models (proc MIXED) with individual as a repeated subject effect, and fixed effects of dose, time and dose*time. We compared average flight speed between doses using linear mixed-effects models. To account for any instances of multiple tower to tower measurements recorded for a single bird, individual bird

was included as a random factor nested within the fixed effect of dose. Tests for differences between means were adjusted for multiple comparisons using the Tukey-Kramer method. The significance level was set at $\alpha = 0.05$.

To estimate the effect of imidacloprid dose on stopover duration, we used semiparametric regression based on the Cox proportional hazards modeling framework using the PHREG procedure in SAS, which allows for assessing the influence of time-dependent covariates (i.e. covariates that change on each possible day of departure) on the probability of departure (*48*). To account for multiple birds with the same stopover duration, we used the TIES = EXACT option, which assumes time is continuous and that there is a true but unknown ordering of tied stopover durations. The resulting hazard ratios (HR = e^β) indicate the probability of departure associated with each variable. HRs >1 indicate greater probability of departure and shorter stopover durations, and HRs <1 indicate lower probability of departure and longer stopover durations. Data were entered using the counting process syntax to obtain the average predicted probability of departure for each dose group over time, using the DIRADJ and GROUP options in the BASELINE statement.

Both extrinsic (e.g. weather) and intrinsic (e.g. pre-dosing fat stores, time held in captivity) factors unrelated to imidacloprid dose could influence stopover duration, and were included as covariates in candidate models. Weather conditions on each possible day of departure were included as a time-dependent covariate. Tailwind and temperature were highly positively correlated (Pearson's r = 0.884, *p* < 0.0001); therefore, a weather index was calculated as the first principle component (PC1) of a PCA of tailwind and temperature. PC1 explained 94.2% of the variance in weather. Pre-dosing fat was included to control for individual variability in migratory readiness not associated with dose. Days in captivity was evaluated to account for possible effects of time in captivity before being screened into the dosing study. Akaike's Information Criterion corrected for small sample size (AICc) was used to rank candidate models (Table S3). Intrinsic covariates (fat, time in captivity) were included in all models except the null, and candidate models tested for the influence of dose, weather, and the interactions between dose*fat and dose*weather on stopover duration. Mean ± SE parameter estimates, hazard ratios, and 95% confidence intervals (CIs) for the top models (ΔAICc ≤ 2) are reported (Table S4). Variables in the top model were considered important if the 95% CI did not overlap one.

The bearing and great-circle distance travelled between towers was calculated using the "Geosphere" package in R. Rayleigh's uniformity test was used to test for significant mean orientation within each dose group, and Watson Williams F-test was used to compare mean orientations across dose groups.

Table S1. Comparison of Imidacloprid low (1.2 mg IMI·kg bm^{-1}·day^{-1}) and high (3.9 mg IMI·kg bm^{-1}·day^{-1}) doses to potential exposure through seed consumption based on current application rates in Canada[a] and the US[b]. Estimates are based on an average white-crowned sparrow body mass of 27 g, and average seed consumption 7.9 g·day^{-1} [c]

	Cereals[e] US	Cereals Canada	Canola US	Canola Canada	Corn US	Corn Canada	Sunflower US
Average mass of individual seed (g)[d]	0.035	0.035	0.003	0.003	0.38	0.38	0.05
Application rate	up to 2.4 fl oz/ 100lbs	up to 30 g a.i./ 100kg seed	up to 25.6 fl oz/ 100lbs	802 g a.i./ 100kg seed	up to 1.34 mg/ kernal	48 g a.i./ 80,000 seeds	up to 0.5 mg a.i./ seed
mg active ingredient (a.i.)/g seed	0.93	0.3	9.97	8.02	3.53	1.58	10
mg a.i. per individual seed	0.033	0.011	0.03	0.024	1.34	0.6	0.5
Number of seeds consumed per day (if 100% of diet)	226	226	2633	2633	21	21	158
mg IMI per day if diet 100% treated seeds	7.5	2.5	79	63.2	28.1	12.6	79
Number of seeds that contain equivalent to high dose (0.105 mg for a 27 g bird)	3.2	9.6	3.5	4.4	0.1	0.2	0.2
% of diet made up of treated seeds to consume equivalent to high dose	1.42	4.25	0.13	0.17	0.48	0.95	0.13
Number of seeds that contain equivalent to low dose (0.032 mg for a 27 g bird)	1	2.9	1.1	1.4	0.02	0.1	0.1
% of diet made up of treated seeds to consume equivalent to low dose	0.44	1.28	0.04	0.05	0.1	0.48	0.06

[a]Health Canada, Imidacloprid: Proposed Re-evaluation Decision. Pest Management Regulatory Agency PRVD2016-20 appendix IIa.
[b]Gaucho 600 flowable, US EPA reg 264-968
[c]food consumption values from (*49*)
[d]Seed masses from (*50*)
[e]wheat, barley, oats, rye, triticale

Table S2. Comparison of mean (SE) body mass, body fat, and lean mass values in birds exposed to a vehicle control, low (1.2 mg IMI·kg bm^{-1}·day^{-1}) or high (3.9 mg IMI·kg bm^{-1}·day^{-1}) imidacloprid. Pre-dosing measures were taken the day before dosing, and post-dosing measures were taken ~6 hours after exposure (see Figure 1 for timeline). P-values for post-hoc comparisons of least squares means are reported for pair-wise pre-dosing vs. post-dosing measures within treatment.

Measurement	Treatment	Pre-dosing	Post-Dosing	% Change	*p*-value
	Control	30.3 (1.1)	29.8 (1)	-1.5	0.156
Body Mass (g)	Low	31.4 (0.4)	30.5 (0.5)	-3.0	0.005
	High	30.1 (0.9)	28.4 (0.9)	-5.9	<.0001
	Control	4.1 (0.7)	4.0 (0.7)	-0.7	0.521
Fat (g)	Low	4.3 (0.5)	3.8 (0.5)	-9.3	0.01
	High	4.2 (0.4)	3.5 (0.4)	-17.1	<.0001
	Control	23.1 (0.5)	22.8 (0.4)	-1.2	na[a]
Lean Mass (g)	Low	24.0 (0.4)	23.5 (0.3)	-1.8	na
	High	22.6 (0.5)	22.2 (0.5)	-1.6	na

[a]Within-treatment pairwise comparisons for pre-dosing vs. post-dosing not conducted for effects that did not have a significant dose*time interaction

Table S3. Body mass data from a previous captive study (*10*) in white-crowned sparrows demonstrating acclimation to captivity, and changes in mean (SE) body mass of birds exposed to imidacloprid (IMI) or a vehicle control (sunflower oil). During the two-week acclimation period, white-crowned sparrows either maintained or gained body mass, suggesting they were not experiencing a strong stress response to captivity at the time they were first dosed, but nevertheless had a rapid and strong negative response to imidacloprid. "Capture" mass represents body mass at capture, "post-acclimation" mass represents body mass at ~2 weeks post-capture, prior to any dosing, and "post-dose" masses represent mass 24 hours after each dose. Control birds maintained body mass for the duration of dosing ($p > 0.213$). IMI caused a significant reduction in body mass (dose*time $p < 0.0001$) in both the low (4.0 mg IMI·kg bm^{-1}·day^{-1}) and high (10.7 mg IMI·kg bm^{-1}·day^{-1}) IMI exposure groups starting after the first dose (post-acclimation vs. post dose 1 mass: low dose $p < 0.001$, high dose $p = 0.0001$) and continued throughout the dosing period (post-acclimation vs. post dose 3 mass: low dose $p < 0.0001$, high dose $p < 0.0001$).

Treatment	Body mass (g)				
	Capture	Post-acclimation	Post dose 1	Post dose 2	Post dose 3
Control	25.8 (0.8)	27.7 (0.6)	27.4 (0.7)	27.5 (0.7)	26.8 (0.8)
Low	28.3 (0.9)	28 (0.6)	26.1 (0.6)	24.6 (0.7)	23.2 (0.9)
High	26.3 (0.6)	28.4 (0.8)	25.7 (0.8)	23.8 (0.8)	21.1 (1.4)

Table S4. Model selection results for Cox proportional hazards regression models estimating the influence of imidacloprid exposure as well as intrinsic (fat stores, time in captivity) and extrinsic (weather) covariates on stopover duration of white-crowned sparrows following a single oral dose of 0, 1.2 or 3.9 mg·kg bm^{-1}·day^{-1} of imidacloprid during spring migration.

Model	k[a]	AICc [b]	ΔAICc [c]	ω[d]
Stopover ~ dose + days in captivity + fat + weather	5	68.30	0	0.968
Stopover ~ days in captivity + fat + weather	3	75.60	7.30	0.025
Stopover ~ dose + days in captivity + fat + weather + dose*fat + dose*weather	9	78.71	10.41	0.005
Stopover ~ dose + fat + days in captivity	4	81.33	13.03	0.001
Stopover ~ fat + days in captivity	2	88.01	19.70	<0.001
Null model	0	97.44	29.14	<0.001

[a] k: number of estimated parameters in the model
[b] AICc: Akaike's Information Criterion corrected for small sample sizes
[c] ΔAICc: difference from AICc of the best-fit model
[d] ω: AICc model weight

Table S5. Parameter estimates (β) and hazard ratios (HR) for candidate Cox regression models explaining variation in stopover duration of white-crowned sparrows exposed to a single oral dose of 0, 1.2 or 3.9 mg·kg bm^{-1}·day^{-1} of imidacloprid. HRs > 1 indicate increased likelihood of departure and shorter stopover durations, and HRs < 1 indicate decreased likelihood of departure and longer stopover durations. HR confidence intervals (CI) calculated using the profile likelihood method. Variables with CI that do not overlap 1 in bold.

Parameter	β	SE	χ^2	p	HR	HR 95% CL
High dose (vs. Control)	-2.15	0.73	8.55	0.003	0.117	**0.024 - 0.453**
Low dose (vs. Control)	-0.14	0.58	0.06	0.813	0.872	0.272 - 2.754
Pre-dosing fat (g)	0.23	0.14	2.66	0.103	1.261	0.947 - 1.665
Days in captivity	0.88	0.36	6.07	0.014	2.399	**1.261 - 5.129**
Weather index (PC1)	0.86	0.24	12.5	0.0004	2.356	**1.514 - 3.995**