Effects of a Neonicotinoid Insecticide and Population Density on Behaviour and Development of Wood Frogs (*Lithobates sylvaticus*)

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Abstract

Amphibians have been facing global declines over the last decades due to direct and indirect effects of anthropogenic activities. One of the leading causes is environmental contamination, particularly that of waterbodies which are used by many amphibian species for reproduction, development, and adult life. An important source of contamination comes from agricultural runoffs of pesticides such as neonicotinoids, which are known to alter anuran survival, behaviour, predation stress response, and development. However, few studies have investigated the possible interactions between neonicotinoids and natural environmental stressors which could alter the strength and direction of observed neonicotinoid effects. This study investigated how a concentration of imidacloprid (a neonicotinoid) measured in surface waters interacted with high population density, an important environmental stressor, to influence behaviour and development across metamorphosis in wood frogs (*Lithobates sylvaticus*) known to breed in agricultural landscapes. I reared tadpoles in a fully crossed design experiment, between two densities (0.33 and 1 tadpole/L) and clean *vs* contaminated water (10 µg/L imidacloprid). Behaviours were measured in the absence and presence of predation cues using open-field tests at three distinct developmental stages, up to the metamorph stage. I found that imidacloprid did not interact with population density or independently affect behaviours in the absence of predation cues. However, individuals raised at high density compared with low density were more active at an early developmental stage but less active at metamorphic climax. Furthermore, both density and imidacloprid independently decreased the natural behavioural response (i.e., "freezing") of tadpoles to predation cues. Both treatments also slightly accelerated metamorphosis while only density altered final mass at metamorphosis. Finally, I found that distance travelled was weakly repeatable between aquatic stages but not repeatable across metamorphosis, a pattern that was

not affected by treatments. This study provides novel insights on the ecotoxicology of imidacloprid in the presence of a natural stressor, highlighting the importance of including behavioural assays and stressors in studies of amphibian ecotoxicology.

Résumé

Les amphibiens sont confrontés à un déclin mondial depuis les dernières décennies en raison des effets directs et indirects des activités anthropiques. L'une des principales causes est la contamination de l'environnement, en particulier celle des plans d'eau qui sont utilisés pour la reproduction, le développement et la vie adulte de nombreuses espèces d'amphibiens. Une source importante de contamination provient des ruissellements agricoles de pesticides tels que les néonicotinoïdes, qui sont reconnus pour affecter la survie, le comportement, la réponse au stress de la prédation et le développement des anoures. Cependant, peu d'études ont examiné les interactions possibles entre les néonicotinoïdes et les facteurs de stress environnementaux naturels qui pourraient modifier la force et la direction des effets observés des néonicotinoïdes. Cette étude a examiné comment une concentration d'imidaclopride (un néonicotinoïde) mesurée en eau de surface interagissaient avec la densité de population, un facteur de stress environnemental important, pour influencer le comportement et le développement au cours de la métamorphose chez les grenouilles des bois (*Lithobates sylvaticus*) reconnues pour se reproduire dans des paysages agricoles. J'ai étudié des têtards dans une expérience à plan croisé complet, entre deux densités (0,33 et 1 têtard/L), en eau propre et en eau contaminée (10 µg/L d'imidaclopride). Les comportements ont été mesurés en absence et présence d'indices de prédation à l'aide de tests "open-field" à trois stades de développement distincts, jusqu'au stade

de grenouille juvénile. J'ai constaté que l'imidaclopride n'interagissait pas avec la densité de population et n'affectait pas indépendamment les comportements en l'absence d'indices de prédation. Les individus élevés à haute densité par rapport à ceux élevés à faible densité étaient plus actifs à un stade précoce de développement, mais moins actifs au climax métamorphique. De plus, la densité et l'imidaclopride ont indépendamment diminué la réponse naturelle de diminution de l'activité des têtards aux indices de prédation. Les deux traitements ont également légèrement accéléré la métamorphose alors que seule la densité a modifié la masse finale à la métamorphose. Finalement, j’ai constaté que la distance parcourue était faiblement répétable entre les stades aquatiques, mais non répétables au cours de la métamorphose, un résultat qui n'était pas affecté par les traitements. Cette étude fournit de nouvelles informations sur l'écotoxicologie de l'imidaclopride en présence d'un facteur de stress naturel, soulignant l'importance d'inclure des mesures de comportement et des facteurs de stress dans les études sur l'écotoxicologie des amphibiens.

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Contents

Introduction

Amphibian populations have been facing global declines over the past decades and human activities, such as habitat destruction and contamination, are a leading cause (Sparling 2002; Vié et al. 2009). Understanding the effects of habitat contamination on amphibians is crucial yet complex, since contaminants can have both direct and indirect effects (i.e., trophic cascades, behavioural and developmental effects which decrease fitness; Collins 2010; Peterson et al. 2017). Many amphibian species are dependent on waterbodies for their larval and/or adult life where contamination can be particularly harmful (Sparling 2002; Vié et al. 2009). Agricultural practices are an important source of water contamination through the use of numerous distinct fertilizers and pesticides (Sparling 2002; Blaustein et al. 2003). Although acceptable concentrations of contaminants in the environment can be determined from survival assays of non-target organisms under laboratory conditions, these may not always accurately reflect natural processes, where stressors occur simultaneously and endpoints are not limited to survival but extend into life-history and behavioural traits which may ultimately influence fitness (Peterson et al. 2017).

Neonicotinoids

Neonicotinoids have been dominating the worldwide insecticide market over the past decades (Jeschke and Nauen 2008). These neuro-active insecticides agnostically bind to post-synaptic nicotinic acetylcholine receptors in the central nervous system of insects, causing membrane depolarization, muscle tetanus, cell damage, and death of insects (Yamamoto et al. 1998; Mehlhorn et al. 1999; Matsuda et al. 2001; Charpentier et al. 2014). Their popularity in

agriculture can be attributed to their effectiveness on managing insect pest species, low application rates, the variety of application methods (seed coating, foliage applications, soil application, etc.), and their systemic activity within the plant due to their hydrophilic nature (Jeschke and Nauen 2008; Charpentier et al. 2014; Morrissey et al. 2015). From an ecological perspective, neonicotinoids' hydrophilic nature is at the root of their potency as waterway contaminants (Morrissey et al. 2015). They have been found to contaminate waterways through runoff and leaching events following rain and snowmelt, but also through such means as treated plant/seed decay and soil or spray drift into waterbodies (Morrissey et al. 2015; Main et al. 2016). Surface water concentrations of imidacloprid (a neonicotinoid) have been known to reach up to 320 µg/L, which is much greater than environmental limits set by various governments, such as 0.23 µg/L in Canada, even though concentrations up to 10.4 µg/L are still observed in this country (Anderson et al. 2015; Morrissey et al. 2015; Struger et al. 2017). The persistent nature of neonicotinoids is also problematic; their condition-dependent half lives range from days in water to 3.5 years in soil depending on the type of neonicotinoid, which increases the risk of chronic exposure in amphibians who use waterbodies fed by agricultural landscapes for larval development and/or adulthood (Morrissey et al. 2015; Main et al. 2016; Sievers et al. 2018, 2019). Chronic exposure risks are also increased when multiple crops with different growing seasons, requiring different application times and rates, are found in the same locations (Morrissey et al. 2015; Struger et al. 2017).

Developmental and behavioural endpoints

The effects of neonicotinoids have been evaluated in some amphibian species with varying results depending on overall study design (type of neonicotinoid used, time and concentration of exposure, species of amphibian, etc.). For example, in spotted marsh frog (*Limnodynastes tasmaniensis*), Sievers et al. (2018) found a significant increase in tadpole mortality after acute (24h) exposure at Gosner (1960) stage (Gs) 22 to low imidacloprid concentrations of 0.5 µg/L (five to six orders of magnitude smaller than the LC50 found in other studies; Feng et al. 2004; Pérez-Iglesias et al. 2014; Sievers et al. 2018). However, a study on wood frog (*Lithobates sylvaticus*) tadpoles chronically exposed to 10 and 100 µg/L imidacloprid for six weeks reported increased survival and slightly delayed metamorphosis compared with controls (Robinson et al. 2017). Also, Robinson et al. (2019) found no effect of clothianidin or thiamethoxam (neonicotinoids) at concentrations of 2.5 and 250 µg/L on leopard frog (*Lithobates pipiens*) or wood frog tadpole survival and development across metamorphosis. However, further analyses of Robinson et al. (2019) by Gavel et al. (2019) found that chronic exposure to these neonicotinoid treatments may have caused anemia in wood frogs, which if maintained (chronic condition) could result in tissue damage, increased stress, and lowered fitness later in life.

Empirical studies also indicate that neonicotinoids can affect amphibian behaviours potentially through effects on hormone levels. For example, Gavel et al. 2019 reported significantly lower levels of corticosterone for tadpoles exposed to 250 µg/L of thiamethoxam. They suggested that neonicotinoids may impair the natural functioning of the hypothalamic-pituitary-adrenal (HPA) stress axis, which could impair amphibian's ability to respond to stressors in their environment. Lee-Jenkins and Robinson (2018) found, for instance, that chronic exposure of wood frog tadpoles to 10 µg/L imidacloprid compromised juvenile frogs' ability to

perceive and/or respond to predator threats three weeks after exposures were terminated. Similarly, Sievers et al. (2018) found that spotted marsh frog tadpoles exposed to imidacloprid and iron-imidacloprid mixtures failed to perceive and avoid predator cues after acute (24h) exposures to concentrations of 0.25 and 0.5 µg/L. They also detected a decrease in the ability to escape a simulated predator (measured as time to travel 25 mm when prodded) in imidacloprid exposed tadpoles (Sievers et al. 2018). In a natural setting, failure to recognize and react to presence of the predator itself, or alarm cues from conspecifics, increases risk of predation (Moore et al. 2015; Polo-Cavia et al. 2016). Therefore, exposures to neonicotinoid could indirectly affect tadpole survival through effects on behaviour. However, behavioural traits are highly labile, being variable through development and affected by environmental conditions (Réale et al. 2007, 2010). Therefore, it remains important to evaluate neonicotinoid effects in chronically exposed tadpoles under ecologically realistic conditions that vary in one or more natural environmental factors.

Amphibian personality

Given their broad spectrum of actions, neonicotinoids could also affect other fitness-related behaviours linked to personality traits, defined as consistent inter-individual differences in expression of behaviours (Dingemanse and Réale 2005; Réale et al. 2007, 2010; Peterson et al. 2017). Personality traits typically fall along five major axes; activity, boldness/shyness, exploration/avoidance, sociability, and aggression, of which the first three have received most attention in amphibians (Réale et al. 2007, 2010; Kelleher et al. 2018). For the few studies that have assessed some of these personality traits after exposure to neonicotinoids, inconsistencies

exits (Gibbons et al. 2015; Shuman-Goodier and Propper 2016; Sievers et al. 2019). For example, Miles et al. (2017) found no effect of clothianidin on leopard frog tadpole activity within a 48h exposure period (Gs unknown), even at high concentrations (1 mg/L). In an acute (96h) exposure study, however, Holtswarth et al. (2019) found that Gs 25 southern leopard frog tadpoles' (*Rana sphenocephala*) distance travelled and mean velocity were negatively affected by increasing concentrations of clothianidin (0 to 6 µg/L). In another study, Gs 22 marsh frog tadpoles had increased erratic swimming and decreased distance travelled following an acute (24h) exposure to 0.25 and 0.5 µg/L imidacloprid (Sievers et al. 2018). Yet, much remains to be determined as to the effects of chronic exposures on tadpole behaviours at multiple developmental stages throughout metamorphosis.

Another aspect of behaviour that has gained increasing interest in recent years relates to whether personality traits are maintained across metamorphosis, and whether environmental factors, such as contaminants, can affect observed patterns of maintenance or dissociation (Réale et al. 2007, 2010; Wilson and Krause 2012a; Urszán et al. 2015a, 2015b; Kelleher et al. 2018). Though many studies have found within-developmental stage repeatability for behaviours along three axes (activity, exploration/avoidance and boldness/shyness; reviewed in Kelleher et al. 2018), very few have investigated whether these behaviours were repeatable across metamorphosis. There are two main predictions regarding how personality should be maintained or changed across metamorphosis (Wilson and Krause 2012a, 2012b; Kelleher et al. 2018). Selection may have acted as to decouple personality before and after metamorphosis when ecological niches differ greatly between developmental stages, as is often the case for amphibians. However, if personalities are linked to inflexible genetic and physiological mechanisms, they may be maintained across metamorphosis, even if disadvantageous in a new

ecological niche (Wilson and Krause 2012a, 2012b; Kelleher et al. 2018). There is still little evidence supporting either prediction. Wilson and Krause (2012b) found that activity (time spent moving) and exploration (latency to first movement in a novel area) were repeatable across metamorphosis (between a larval stage and juvenile frogs) in the lake frog (*Rana ridibunda*), supporting the maintenance of personality. By contrast, Brodin et al. (2013) found that exploration (area covered in a novel arena) and boldness (time to exit a refuge) were not repeatable across metamorphosis (between Gs 37 and juvenile frogs) in the common frog (*Rana temporaria*), supporting the decoupling theory. Concerning effects of the environment on maintenance of personality, some studies found that personality traits in agile frog (*Rana dalmatina*) tadpoles were only consistent between more developed tadpoles, when tadpoles had prior experience with a stimulus, or when tadpoles were raised in more complex environments (i.e. in the presence of social and predation cues; Urszán et al. 2015a, 2015b). Furthermore, considering that contaminants can alter stress hormone levels (Gavel et al. 2019; Trudeau et al. 2020) which are related to behaviour expression and play key roles in the metamorphosis process of amphibians, studying whether contaminants affect the maintenance of personality differences across metamorphosis may shed more light on the indirect effects of contaminants.

Environmental stressors

Another aspect of ecotoxicology that is often overlooked are stressors, such as predator presence, high resource competition, and high population density, which may increase the direct and indirect effects of contaminants on amphibians (Pochini and Hoverman 2017; Sievers et al. 2019). The presence of predator cues has been shown to increase susceptibility of some

amphibian species to agrochemicals such as glyphosate and carbaryl (Relyea 2003, 2005; but see Relyea 2012). High population density by itself has been shown to decrease survival, reduce growth, delay metamorphosis, and increase corticosterone production (Wilbur 1976; Semlitsch and Caldwell 1982; Glennemeier and Denver 2002; Rot-Nikcevic et al. 2005; but see Belden et al. 2007). Density may also alter the negative effects of chemicals, such as increasing the lethality of pesticides (Boone and Semlitsch 2001; Jones et al. 2011; Ortiz-Santaliestra et al. 2012). Understanding the vulnerability of amphibians to pesticides with the addition of varying stress levels may provide more realistic insights of their sensitivity to chemicals in the wild.

Objectives

This project aims to determine how behaviours, including predation cue responses, and developmental endpoints are affected in wood frogs chronically exposed to an environmentally relevant concentration of imidacloprid at different population densities. I performed a fully crossed experimental design between two concentrations of imidacloprid and two population densities to investigate their interactive effects on traits of interest using repeated individual measurements across metamorphosis. Given previous studies on the effects of neonicotinoids and other pesticides, I expected imidacloprid to impair behaviours related to activity and boldness, and negatively affect responses to predator cues (Shuman-Goodier and Propper 2016; Lee-Jenkins and Robinson 2018; Sievers et al. 2018, 2019; Holtswarth et al. 2019). More precisely, I expected a decrease in distance travelled, an increase in latency to first movement and central area time (potentially boldness; Carlson and Langkilde 2013; Denoël et al. 2013), and a failure to respond to conspecific alarm cues (i.e., failure to freeze and/or reduce activity after

cues are added; Richardson 2001; Ferrari et al. 2007; Ferrari and Chivers 2009). I also expected that distance travelled (a proxy of activity) would be repeatable across metamorphosis, more so under the imidacloprid and high population density treatments as seen in other studies where variation in environmental variables can generate consistent among-individual differences in expressions of behaviours which are not expressed in simplified environments (Wilson and Krause 2012b; Urszán et al. 2015a, 2015b). Finally, I also expected that high densities and imidacloprid would increase metamorphosis time and that density would decrease mass at metamorphosis (Wilbur 1976, 1977).

Materials and Methods

Wood frogs are widespread across North America and are commonly used as biomarkers of aquatic environment health (Relyea et al. 2005; Lanctôt et al. 2013; Edge et al. 2014; Dananay et al. 2015).They breed in aquatic environments within agricultural land, and are therefore at risk of exposure to neonicotinoids (Mason et al. 2013; Robinson et al. 2017).

Experimental design

This study was conducted during the spring and summer of 2019 in southern Quebec, Canada (45°21'40.6"N; 71°50'32.2"W). The experiment consisted of two concentrations of imidacloprid (0 and 10 µg/L) and two densities (low and high; 0.33 and 1 tadpole/L), creating four treatments with three replicates each (n = 12 tanks). I used 416 L cattle tanks that were inserted 30 cm into the ground to control temperature fluctuations. The tanks were filled with 150 L aged tap water and maintained at this volume throughout the experiment (see *Supplemental Figure 2A*). The

tanks were capped with chicken wire fence and mosquito net lids to provide shade and exclude animal intruders. The substrate was composed of dead deciduous leaves and tadpoles were fed *ad libitum* boiled lettuce replaced every three days (Semlitsch and Caldwell 1982; Calsbeek and Kuchta 2011; Wilson and Krause 2012b). Each tank contained 25 meshed cups made of 1 mm^2 aluminum mesh folded into an open top cylinder (diameter = 9 cm, length = 20 cm). Cups were attached to untreated wooden dowels and submerged 15 cm into the tank providing a swimming volume of approximately 1 L and access to the majority of the water column and to the water's surface (see *Supplemental Figure 2B*). Each cup contained two dried deciduous leaves for refuge. Cups served as a means of tadpole identification permitting repeated measures of the same individual across metamorphosis. Cups also allowed for the sharing of both chemical and visual cues from conspecifics which are important density cues for tadpoles (Rot-Nikcevic et al. 2005, 2006; Sutherland et al. 2009).

Tadpoles were obtained from 14 wood frog egg masses collected on May 5th from an ephemeral pond adjacent to the study site. Egg masses were transferred into a rearing tank which was identical to the experimental tanks. Individuals began hatching on May 6th and were haphazardly selected and distributed among experimental tanks on May 16th (Gs ~25) as either free tank swimmers (25 or 125 tadpoles for low- and high-density treatments) or cupped focal individuals (25 per tank, n = 300). A total of 1200 tadpoles were distributed among experimental tanks. Tadpoles were left to complete metamorphosis within tanks and focal individuals had their behaviours measured via open-field tests conducted at Gs ~30, 42, and 45/46, which represent two aquatic stages and a mostly terrestrial stage, respectively. When forelimb emergence began (Gs 42), the tops of the cups were crimped to prevent escapes, and 1 cm thick slices of weathered PoolNoodle® were added to the tanks and cups so that metamorphs could exit the water to rest if

needed. Metamorphs were removed as they completed metamorphosis (end of tail resorption; Gs 45/46). All frogs were then brought to the lab for euthanasia and final measurements. Frogs were anesthetized in a 0.02% buffered MS-222 bath and euthanized in a 0.2% MS-222 bath followed by pithing (Robinson et al. 2017). Frogs were weighed using an analytical balance (Adam Equipment PW124 Analytical Balance). All manipulations and procedures were approved by Bishop's University Animal Care and Biosafety Committee (#102009) and by the University of Ottawa (#3138).

Contaminant and predation cue preparation

The 10 µg/L imidacloprid concentration was obtained by diluting 0.625 mL of Admire (240 g/L of imidacloprid; Bayer CropScience; CAS no. 138261-41-3) in 1 L of reverse osmosis water to obtain a stock solution with a concentration of 0.15 g/L. The stock solution was kept in an amber glass bottle in the dark at 4ºC to avoid degradation (Robinson et al. 2017), and was tested to determine its nominal concentration, which was 0.145 g/L. The tanks were inoculated in two pulses on May 16th, before tadpoles were introduced, and three weeks later, on June 6th. The tanks either received 10.3 mL of the stock solution or 10.3 mL of reverse osmosis water stored under identical conditions. I collected 500 mL water samples from each tank on two occasions, one hour after inoculation (May 16th and June 6th) and at the end of the experiment (July 18th). I also collected samples from the natural breeding sites where egg masses were collected, and from the tap water to verify that these were not contaminated by imidacloprid. Samples were stored in amber bottles stored at 4ºC before being analyzed by the Laboratory Services at the National Wildlife Research Centre, Environment and Climate Change Canada (Ottawa, ON,

Canada) using techniques described in Robinson et al. (2017). I measured tank temperatures every morning within a 10 to 15 minute time period using a standard thermometer, and the pH of my samples were measured using an Accumet Excel XL20 pH/Conductivity Meter. From these tests I determined that clean water tanks were not contaminated, concentrations (for treated tanks) and temperatures did not differ between treatments or tanks, and that pH was slightly lower for low density treatments (see *Supplemental Materials* for more details).

The predation cues used in behavioural tests (see below) were a mixture of aged tap water and alarm cues produced by conspecifics (Fraker et al. 2009). The alarm cue solutions were prepared using a random sample from 200 tadpoles which were kept and maintained in the rearing tank after initial distribution of experimental tadpoles. Tadpoles were poked in the tail muscle for ten seconds at 5 different locations using a 26.5 gauge hypodermic needle while being gently held in place in aged tap water using pipette tips (Fraker et al. 2009). Two tadpoles were needed per 100 mL of predation cue and were kept in the cue water for 20 minutes before being returned to their tank. The predation cue water was prepared daily, kept in a cool shaded area during use, and was mixed before being sampled.

Behavioural tests

Three open-field tests were conducted at Gs ~30, 42, and 45/46 (n = 280, 265, and 150 respectively). The first behavioural tests took place over two days (June 15th and 16th) to maintain similar developmental stages between individuals (Gs ~30). The second tests were all conducted as individuals reached Gs 42, between June 30th and July 16th, and the third (terrestrial) tests took place at Gs 45/46, between July 3rd and 18th. Individual's behaviours were

monitored using a mounted digital camera (Panasonic Lumix DMC-ZS7; 12.1-megapixel resolution, 1/2.33-inch CCD, 12x optical zoom Leica DC Vario-Elmar lens). Aquatic tests were conducted on four tadpoles at a time held individually in arenas constructed from white plastic buckets (diameter = 25.4 cm, height = 6.4 cm; see *Supplemental Figure 1*). A smoothed concave ridge was added with aquarium safe silicone to the corner of each arena that was closest to the camera to prevent individuals from entering this blind spot. Markings were also added one inch away from the bucket's edge in the four cardinal directions and in the center to aid in the distance calibration and zone delimitation in EthoVision (Noldus et al. 2001). Arenas were filled to a depth of 1.5 cm aged tap water to limit vertical movements that could not be measured. Terrestrial tests were performed in a wooden 94.6×87.9×25cm open-field arena painted white with aquarium safe paint, with an added black grid on the floor for movement tracking. The arena was moisturized with aged tap water using a spray bottle before tests to prevent frog desiccation and was wiped between trials to remove any chemicals left by previous tests subjects. In order to provide controlled light condition across the arena, a wall/blind was used for certain tests.

Aquatic tests were filmed within a 63.5×63.5×65.4cm closed wooden box lit with led lights to standardize testing environments by providing steady lighting and preventing external stimuli like wind or insects (see *Supplemental Materials* for more details). Arenas were always placed in the same location and orientation using markings on the box's floor. The ceiling of the box had a hole for the camera aperture and four additional holes above the center of each arena, where four funnels were inserted and used for the addition of predation cues. Prior to aquatic tests, focal individuals were decanted individually from their respective cups into white plastic cups containing the same aged tap water as the arenas. They were then placed inside a cardboard

box, lit in the same manner as the closed wooden box for acclimation. Following five minutes of acclimation, individuals were introduced into the test arenas.

Aquatic open-field trials were comprised of two stages, where behaviours were measured in the absence and presence of a predation cue. The initial stage lasted two minutes following an initial ten seconds of acclimation from the moment the open-field box doors were closed. Then, 10 mL of alarm cue water (see above) was added from the top to stimulate a predation cue response (Fraker et al. 2009). The predation response stage lasted two minutes, also following a 10-second acclimation period to allow the cue to spread and for any water surface disturbances to cease, allowing for proper tracking. At the end of each trial, focal individuals were placed back in their respective cups and returned to their respective tank once all cupped individuals within a tank had been tested. Arenas were rinsed and refilled with clean aged tap water between trials. Terrestrial open-field tests only measured behaviours in the absence of predation cues, starting 10 seconds from the moment an individual was released in the center of the arena.

Video analyses

Videos were all analyzed in EthoVision (Noldus et al. 2001) where central zones (64% of the area) were drawn and distances calibrated. In the case of aquatic arenas, the center zone was a 20.3 cm diameter circle, while the terrestrial arena's central zone was a 76.4×69.7 cm rectangle, leaving a 2.55 cm and 9.1 cm thick border for aquatic and terrestrial tests, respectively. The perspective of the camera had to be corrected for in the terrestrial tests using Lightworks (version 2020.1) program because videos were taken at an angle and from a distance. EthoVision was used to measure total distance travelled, latency to first movement, and time spent in the center

of the arena in both aquatic and terrestrial tests, including predation cue response tests for aquatic tests.

Statistical analyses

All statistical analyses were conducted in R 3.6.3 (R Core Team 2020). Treatment fixed effects (density and imidacloprid; low/high and absent/present respectively) were evaluated as centered continuous variables (assigned a value of -0.5 and 0.5 for each respective level) to determine the significance and direction of fixed effects independent of interactions (Schielzeth 2010). Nuisance variables included water/air temperature (ºC), cloud cover (cloudy *vs* sunny), Julian date (from May 1st), and time of day (minutes from midnight). Aquatic tests also included acclimation time, arena ID, and dorsal surface area (only for Gs ~30 as a proxy for body mass; Davis et al. 2008), while terrestrial tests included blind position (three levels) and final mass. As for dependent variables, distance travelled was first square-rooted to achieve normality of residuals and then scaled to a mean of 0.126 and a variance of 0.969. Latency to first movement and central time were converted to proportional data by dividing values by the maximal duration of the test (120s) to correct for violations of normality of residuals and heteroscedasticity assumptions. For development analyses, dependent variables included final mass (log-transformed and scaled to a mean of 0.907 and a variance of 0.706), metamorphosis time, and survival measured as the proportion of all tadpoles (cupped and free swimming) who had survived to or completed metamorphosis prior to the final day of testing (July 18, 2019, when all cupped individuals had reached Gs 45/46). I included replicate tank and trial number (grouping of four tadpoles in each aquatic trial) as random effects in the initial mixed-model approach

using packaged lme4 and lmerTest (Bates et al. 2015; Kuznetsova et al. 2017). However, these were non-significant and were removed from further analyses. Individual identity (ID) was also included as random effect for repeatability analyses. When appropriate, I used stepwise backward model selection, verified with AIC/likelihood-ratio test (LRT) model selection, to estimate *P*-values and obtain the final models presented in results (see *Supplemental Material* for full models).

The repeatability of distance travelled across metamorphosis was calculated using the rptR package (Stoffel et al. 2017). I measured overall repeatability (Gs ~30, 42, and 45/46), repeatabilities between all stages (Gs ~30 and 42, Gs 42 and 45/46, and Gs ~30 and 45/46), and whether these repeatabilities differed among treatments. Tadpole ID was specified as a random effect, while replicate tank and trial number were excluded since they were not significant in the models. Fixed effects included treatment variables, their interaction, and nuisance variables when measured for all tests. I specified 1000 bootstrapping and permutations per model. The repeatability of response to predation cues was analyzed in the same fashion with the inclusion of distance travelled before the addition of the cue as a fixed effect to account for differences in the expression of the behaviour between individuals (i.e., the analysis informs us on the behavioural response of tadpoles before vs after the predation cues). The quasi-binomial distribution of proportional latency to first movement and central time prevented us from testing for their repeatabilities.

I then used linear models (LM; for distance travelled) and generalized linear models (GLM with quasi-binomial distributions for latency and central time) to test the effects of pesticide and population density on the three behaviours measured for each Gs separately. Behaviours were evaluated by Gs based on the overall lack of significant repeatability of

distance travelled (activity) between developmental stages (see *Results*). Furthermore, since replicate tank and trial number were not significant in my initial mixed model approach (based on model selection), they were excluded from the models. Fixed effects included treatments, their interaction, and test-specific nuisance variables. Interactions between treatment variables and variables related to size (surface area at Gs ~30, and mass at Gs 45/46) were also included, up to three-way interactions. The effect of pesticide and population density on predation cue response was measured separately. The predation cue response analyses followed the same format as the pre-predation-cue behaviour analyses, but also included the pre-cue behaviour as fixed effect to account for differences in the expression of the behaviours prior to the addition of the cue, and therefore model the behavioural response to predation cues.

Finally, I tested whether the treatments influenced final mass at metamorphosis, developmental time (emergence date), and survival using packages lme4 and lmerTest (Bates et al. 2015; Kuznetsova et al. 2017). Individuals that did not complete metamorphosis were excluded from mass and emergence time analyses (n = 98). Mass was analyzed using a linear mixed model (LMM) with replicate tank as random effect (n = 989 individuals). Developmental time was analyzed using a generalized linear model (GLM) with a quasi-Poisson distribution to address under-dispersion (random effects cannot be included in quasi-Poisson models, but the significance of Tank was assessed in a GLMM with a Poisson distribution, and it was not significant, p = 0.06, n = 994). Models for mass and metamorphosis time included treatment variables, rearing condition (free *vs* cup, hereafter referred to as *cup*) treated as centered continuous fixed effects, and their interactions, up to the three-way interactions. Finally, ANOVAs were conducted to determine whether survival differed between tanks or treatments.

Results

Repeatability of distance travelled

Distance travelled was repeatable between Gs ~30 and 42 (R ± se = 0.150 ± 0.061, CI = 0.043 – 0.284, $p = 0.012$) and between all stages (Gs ~30, 42, and 45/46; R ± se = 0.092 ± 0.045, CI = 0.010 – 0.187, $p = 0.039$). However, it was not repeatable between Gs ~30 and 45/46, or between Gs 42 and 45/46 (all $p > 0.05$; see *Supplemental Table 9*). Further analyses by treatment revealed that distance travelled was only significantly repeatable between these stages for the low-density groups when both imidacloprid treatments were included (for Gs ~30 and 42 R ± se = 0.209 ± 0.084, CI = 0.063 – 0.399, $p = 0.013$; for Gs ~30, 42 and 45/46 R ± se = 0.134 ± 0.064, CI = 0.027 – 0.277, $p = 0.029$). Distance travelled in the presence of predation cues was not significantly repeatable between Gs ~30 and 42 under any condition (all $p > 0.05$).

Behavioural endpoints

Individuals raised at high density travelled significantly further and tended to have shorter latencies to first movement than low-density individuals at Gs ~30 (Table 1). By contrast, Gs 42 tadpoles raised at higher density travelled significantly shorter distances than their low-density counterparts and had significantly longer latencies to first movement (Table 1). Finally, for Gs 45/46, there was a significant "density × mass" interaction, such that latency to first movement decreased with mass at high densities but tended to increase at low densities (Figure 1A). The "density × mass" interaction was also significant for central time, where central time at high density decreased significantly more with mass than at low densities (Figure 1B). For distance travelled, larger individuals traveled significantly further than smaller individuals. In analyses of

predation cue responses, both density and imidacloprid had a significant effect on distance travelled at Gs ~30. Individuals raised at high densities and exposed to imidacloprid travelled further than their low-density (0.201 ± 0.1, t = 2.016, p = 0.045) and clean water (0.277 ± 0.1, t = 2.782, p = 0.006) counterparts. Density and imidacloprid treatments did not significantly differ for latency and central time at Gs ~30 and Gs 42, or distance travelled at Gs 42 (see *Supplemental Materials* for full model estimates).

Developmental endpoints

Analyses of body mass and developmental time revealed significant treatment effects. Final mass at metamorphosis of all individuals (free swimmers and cupped individuals) was significantly related to density (-1.278 ± 0.138, t = 9.284, p < 0.001), cup (-0.451 ± 0.059, t = 7.702, p < 0.001) and their interaction (1.16 ± 0.117, t = 9.901, p < 0.001). Both high population density and being raised in cups significantly reduced body mass at metamorphosis (mean difference of 0.155g (~37.7% decrease) and 0.003g (~1% decrease) respectively), though cup's effect was strongest at low density (mean difference of 0.128g (~28.3% decrease) *vs* 0.01g (~4.1% increase) at high density) as demonstrated by the significant interaction (Figure 2). Imidacloprid had no significant effect on final mass (p = 0.12). For analyses of developmental time of all frogs, imidacloprid exposure (-0.009 ± 0.003, t = 3.343, p = 0.001), high population densities (-0.020 ± 0.003, t = 6.014, p < 0.001), and being raised in cups (-0.014 ± 0.004, t = 3.546, p < 0.001) led to significantly earlier emergence of frogs (mean difference of 0.67, 1.17, and 0.52 days respectively). Finally, there were no differences in frog survival between tanks or treatments (all p > 0.05).

Discussion

In this study I investigated many aspects of the risk that neonicotinoids may pose to amphibians, among which are behavioural effects, response to predation cues, development effects, and interactions with population density, a known stressor in amphibians (Wilbur 1976; Semlitsch and Caldwell 1982; Glennemeier and Denver 2002; Rot-Nikcevic et al. 2005; but see Belden et al. 2007). I found that distance travelled was only slightly repeatable across metamorphosis, markedly between Gs ~30 and 42, and at low population densities. I also found that imidacloprid did not affect distance travelled, latency to first movement, and central area time, but that population density did and differently so depending on the developmental stage. However, imidacloprid and density did independently increase distance travelled in the presence of predation cues at Gs ~30, a sign of a failure to decrease activity in the presence of a predation cue. I also found that imidacloprid and density accelerated metamorphosis and that density decreased mass at metamorphosis. I did not find any treatment effects on survival. Finally, I found no evidence that imidacloprid's effects were altered by population density.

Personality across metamorphosis

The repeatability analyses of distance travelled in a novel environment (activity) revealed a low but significant repeatability between aquatic stages (R=0.15, Gs ~30 and 42) and across metamorphosis (R=0.09, Gs ~30, 42, and 45/46). These results seem to indicate that distance travelled (activity) is repeatable across metamorphosis, however, considering that distance travelled was not repeatable between Gs ~30 and 45/46, or Gs 42 and 45/46, it is possible that the across-metamorphosis repeatability is primarily driven by the one found between Gs ~30 and

42. To verify this, I grouped aquatic stages (Gs ~30 and 42) and calculated their mean distance travelled, which I used to determine the repeatability of distance travelled between aquatic and terrestrial stages. I found no significant repeatability between aquatic and terrestrial stages, supporting my interpretation that distance travelled was not repeatable across metamorphosis. These results thus imply that in wood frogs distance travelled is slightly repeatable between aquatic stages, but not between aquatic and terrestrial stages, supporting the decoupling of personality across metamorphosis theory (Wilson and Krause 2012a; Kelleher et al. 2018). This result is different to that of Wilson and Krause (2012b) who found that activity (time spent moving) and exploration (latency to first movement in a novel area) was repeatable across metamorphosis in lake frogs. However, my result is similar to Brodin et al.'s (2013) study, which found that exploration (area covered) and boldness (latency to exit refuge) were not repeatable across metamorphosis in common frogs. Measures of activity and exploration tend to be repeatable within developmental stages and between similar aquatic stages (Wilson and Krause 2012b; Brodin et al. 2013; Carlson and Langkilde 2013; Urszán et al. 2015b, 2015a; Kelleher et al. 2017, 2018; Koenig and Ousterhout 2018; Chajma et al. 2020). However, if activity is not repeatable between aquatic and terrestrial stages, it may explain why the repeatability I found between Gs ~30 and 42 is low, as Gs 42 represent the metamorphic climax which involves important physiological and associated behavioural changes in preparation for a transition to a terrestrial juvenile (Gosner 1960; Walsh 2010; Touchon et al. 2013). These differences observed between species and behaviours reflect the need to further study how individual differences in behaviours are maintained or lost through amphibian metamorphosis (Wilson and Krause 2012a; Kelleher et al. 2018).

Further analyses of repeatability within my different treatments revealed that only low-density (with both imidacloprid treatments; not significant when separated by imidacloprid treatment due to reduced sample size) had significant repeatability which followed the same pattern as above (R = 0.209, Gs ~30 and 42; R = 0.134, Gs ~30, 42, and 45/46). I performed the same post-hoc analyses as above for this subset of the data and arrived at the same conclusion; the repeatability found across metamorphosis is driven by the one found between aquatic stages. However, despite finding only significant repeatability for the low-density treatment, I cannot conclude that my low-density treatment significantly differed from other treatment due to confidence interval overlap. It does suggest, however, that the repeatability of certain behaviours may be influenced by the environment during early development (Wilson and Krause 2012b; Urszán et al. 2015a, 2015b; Kelleher et al. 2018; Chajma et al. 2020). This is further supported by the lack of significant repeatability for the predator response (distance travelled in the presence of predation cues), which agrees with findings of other studies that show that prior experience with a predator was necessary for such a trait to be repeatable in an anuran amphibian (Urszán et al. 2015a, 2015b).

Effects of imidacloprid and density on behavioural endpoints

I found no evidence that imidacloprid interacted with density or independently affected distance travelled (activity), latency to first movement (boldness), and time spent in the center of the arena (boldness). Thus, it seems that activities related to foraging (activity) and risk taking (boldness) are unaffected by a two-pulse exposure to the relatively high concentration of 10 µg/L imidacloprid. Furthermore, although this study lacks a positive control for imidacloprid (i.e., a

chemical treatment with known effects on amphibian behaviour and development), the results regarding density effects are in the expected directions. This indicates that individuals were reacting appropriately to their environment which reinforces conclusions regarding the lack of imidacloprid effects on measured traits.

Despite imidacloprid's lack of effects on foraging and risk-taking types of behaviours, density had different effects on distance travelled depending on developmental stage. At Gs ~30, individuals raised at high density travelled further than those at low density, while at Gs 42, the inverse took place where high-density tadpoles travelled less far and had greater latencies to first movement. The effects of high density on Gs ~30 tadpoles could be explained by density's natural enhancement of foraging type behaviours (i.e., activity) prior to Gs 42, as measured in other studies (Relyea 2002; Cothran et al. 2013). The opposite effect observed at Gs 42 could relate to differences in growth rates and energy allocations at high densities. If high density individuals had their growth rates diminished (Wilbur 1976, 1977; Semlitsch and Caldwell 1982; Glennemeier and Denver 2002; Relyea 2002; Cothran et al. 2013), they could have smaller energy reserves at their disposal during this crucial time of metamorphosis which involves cessation of foraging and major morphological and physiological changes (Gosner 1960; Walsh 2010; Touchon et al. 2013). This could have lowered their predisposition for activity which would also explain the greater latencies to first movement.

At Gs 45/46, density only affected metamorphs in an interaction with mass, such that individuals with the smallest mass at high density had longer latencies to first movement and central time compared with low-density treatments and larger individuals of either treatment (Figure 1A, B; Table 1). This result may be related to differences in individuals' reaction to the open-field test, rather than differences in performance between treatments. This suggestion is

based on the lack of significant differences in distance travelled between metamorphs of either treatments at similar masses (the density × mass interaction was not significant), or between density treatments themselves. It may be that the physiological changes incurred by high population densities have a more lasting effect on the smallest metamorphs' behaviours, such as enhancing a freeze response in a novel environment. This would explain the greater latencies to first movement and time spent in the center compared to larger froglets of either treatment. However, without direct measurements of stress hormones, this cannot be verified, though it would be interesting to see if future studies obtain similar results and whether these results could reflect effects on important behaviours such as dispersal or foraging.

Responses to predation cues usually take the form of drastic reductions in activity (Richardson 2001; Ferrari et al. 2007; Ferrari and Chivers 2009; Fraker et al. 2009). I found indications that imidacloprid and population density could impair responses at Gs ~30. Indeed, in the presence of the predation cue, tadpoles exposed to imidacloprid or high density travelled further than clean water or low-density tadpoles. This result agrees with the findings of other studies where various imidacloprid concentrations impaired responses to predation cues (Lee-Jenkins and Robinson 2018; Sievers et al. 2018). Increased activity in the presence of predators could lead to increased predation (Werner and Anholt 1993; Skelly 1994; Van Buskirk and Mccollum 2000). In grey treefrog (*Hyla versicolor*), Van Buskirk and McCollum (2000) found that more active tadpoles had higher probabilities of mortality when encountering a predator. Furthermore, using anesthetics to reduce activity revealed that immobile wood frog tadpoles were less likely to be consumed by a predator (Skelly 1994). Imidacloprid may thus indirectly increase mortality in affected populations (but see Carlson and Langkilde 2014; Miles et al. 2017).

Despite the effect of imidacloprid and density on predation cue response at Gs ~30, I found no evidence that exposure to 10 µg/L imidacloprid or high population density affected predation cue responses at Gs 42. A possible explanation could be that Gs 42 is not an optimal stage for measuring predation cue responses. For instance, Touchon et al. (2013) found that Gs 42 red-eyed treefrog (*Agalychnis callidryas*) tadpoles reduced their activity much less in the presence of a predator cue compared with Gs 41 tadpoles. Perhaps they are less responsive to the cue, or the decrease in activity is less strong due to the natural decrease in activity that occurs at this stage (Touchon et al. 2013). Furthermore, the predation cues may not be as relevant for Gs 42 tadpoles since not all anurans respond to injured conspecific cues after metamorphosis (Chivers et al. 1999). Yet, the addition of alarm cues lead to significantly lower distances travelled and greater latencies to first movement at Gs 42 (see *Supplemental Materials* for details of this post hoc analysis), which supports that Gs 42 tadpoles can still perceive and respond to alarm cues. Hence, the lack of effect of the imidacloprid and density treatments on the behavioural response was not due to a lack of perception of the cue. Concerning imidacloprid, an explanation could be that the HPA axis functioning returned to normal following natural degradation of imidacloprid in the tanks (mean concentration closest to days of testing: 0.742 ± 0.094 µg/L; Gavel et al. 2019, but see Van Meter et al. 2014; Iturburu et al. 2017; Crayton et al. 2020). Further investigation of the mechanism of imidacloprid action relating to body corticosterone and predator responses is necessary to explain these results.

Effects of imidacloprid, density, and isolation cups on developmental endpoints

Analyses of mass and developmental time revealed that both imidacloprid and density can alter metamorphosis metrics in wood frogs. Imidacloprid exposed individuals did not differ in final mass but did emerge earlier on average than clean water tadpoles. The lack of an effect on mass is consistent with other studies investigating imidacloprid or other neonicotinoids (Ade et al. 2010; Robinson et al. 2017, 2019). These results seem to indicate that imidacloprid at these concentrations does not represent an important burden in terms of energy devoted to detoxification, foraging ability (when considering the current behavioural analyses), or other processes that could impair growth (Robinson et al. 2017; Trudeau et al. 2020). Other studies have also found that neonicotinoids either delayed metamorphosis (by ~1d) or had no effect (Ade et al. 2010; Robinson et al. 2017, 2019). In a long-term study of wood frog populations, Berven (1990) found that early metamorphosis and increased mass at metamorphosis lead to larger adult sizes, greater survival, earlier age at first reproduction, and increased reproductive output. Considering that final mass was not significantly affected by imidacloprid treatment, earlier metamorphosis may appear to be a beneficial side effect of imidacloprid exposure. However, it is important to note that the effect size is very small (mean difference of ~0.67d; Table 2), as in Robinson et al. (2017), and unlikely to be of ecological importance. The direction of the effect is also inconsistent between studies, even at similar concentrations, suggesting low ecological concern at these environmentally relevant concentrations (Robinson et al. 2017, 2019).

As expected, population density also altered metamorphosis metrics. High densities induced smaller body masses at metamorphosis (mean difference of 0.155g; ~37.7% decrease compared to the mean mass at low density), a known phenomena in anuran amphibian development (Wilbur 1976, 1977; Semlitsch and Caldwell 1982; Berven 1990; Yagi and Green

2016). Higher population density also accelerated metamorphosis (mean difference of ~1.17d). Individuals at low density tend to grow faster and should delay metamorphosis in exchange for increased mass at metamorphosis when the environment is favourable, while high-density individuals may leave the pond as soon as they reach the minimum required size for completion of metamorphosis, yielding smaller masses and earlier emergence (Wilbur and Collins 1973; Wilbur 1976; Alford and Harris 1988; Robinson et al. 2017). Leaving the pond early can be advantageous within high-stress breeding pools, as doing so releases the metamorphs from competition, though the reduced mass at metamorphosis is linked with decreased survival, adult size, and fecundity in wood frogs (Berven 1990).

Beyond the effects of imidacloprid and population density, the present results suggest that cups may have had unintended side effects on wood frog development. Tadpoles raised in cups within low-density tanks had significantly lower final masses compared with free swimmers (mean difference of 0.128g; ~28.3% decrease in body mass), and similar masses within high-density tanks regardless of treatment (mean difference of 0.01g; 4.1% increase in body mass for cupped individuals, Figure 2). Also, cupped frogs emerged earlier than free swimmers (mean difference ~0.5d). An important note is that many frogs (n = 113) managed to escape their cups following Gs 42 due to improper crimping of the lips of the cups at this stage, which most likely diminished the differences between means of cupped and free-swimming individuals. Studies which have varied levels of isolation such that tadpoles were either fully interacting (no partitions) or partially interacting (meshed partitions, comparable to the current study design) have either found no differences between fully and partially interacting treatments or that partially interacting tadpoles had more rapid metamorphosis and smaller masses at metamorphosis (Breden and Kelly 1982; Griffiths and Foster 1998). A potential explanation

could be that cups may have impaired mobility or provided tadpoles with physical cues of the relatively small swimming volume at their disposal (perhaps similar to a pond-drying stress; John and Fusaro 1981; Gomez-Mestre et al. 2013). Considering the relatively large size of the cups I believe that mobility reduction was unlikely to be the cause for the observed effects. A more likely explanation may be that free swimming tadpoles had greater availability of conspecific feces as secondary food source or benefited from social facilitation of feeding by shared breakdown of food into smaller food particles (Breden and Kelly 1982). In the case of high density, however, the effect of the high-density stress may have overpowered the apparent benefits of the social interaction, leading to small differences in mass observed between cupped tadpoles and free swimmers (Figure 2). Fortunately, despite this effect of the cup, I was still able to detect clear density and imidacloprid effects regardless of rearing condition.

Finally, analyses of survival (measured as the proportion of all tadpoles who had survived to, or metamorphosed prior to the final day of testing) indicated that survival did not differ between replicate tanks or treatments. This result adds to the mixed empirical evidence regarding neonicotinoids effects on survival, which have been shown to decrease survival, increase survival or leave it unaffected at ecologically relevant concentrations (Robinson et al. 2017, 2019; Sievers et al. 2018). The variability may be due to differences in study design. For instance, Sievers et al. (2018) found increased mortality in marsh frog tadpoles at Gs 22, while I tested wood frogs starting at Gs ~25. This may highlight important differences in vulnerability of different amphibian species and developmental stages. However, although I did not find any direct effects of imidacloprid on survival, I do provide evidence that predation cue recognition may be affected in early developmental stages, which could increase mortality at those stages. These results highlight the importance of studying behaviours in ecotoxicology and the

importance of further research in this field if we want to reach a consensus on the risks neonicotinoids may pose to amphibians.

Conclusion

This study demonstrates the importance of integrating behavioural endpoints when evaluating the amphibian ecotoxicology of pesticides as these may reveal effects not detected in conventional studies which may have unforeseen negative effects on survival (i.e., through increased predation) in natural settings. I provided evidence that imidacloprid (at the environmentally relevant concentration of 10 µg/L) can attenuate the "freezing" response to predation cues, which could increase predation-related mortalities in natural settings. However, other behaviours related to foraging and risk taking did not appear to be affected. Furthermore, survival did not differ between treatments and effects on developmental time were probably of low ecological concern. I found no evidence that the effect of high population density was enough to alter imidacloprid's effect on survival, development, or behaviours. I also did not find any effects of imidacloprid on the repeatability of distance travelled across metamorphosis, which was overall low and highlighted a potential density effect. Population density did affect predation cue responses and behaviours, and did so differently depending on developmental stages, highlighting the importance of conducting ecotoxicological studies at population densities found in natural populations. Finally, I emphasize the need to include variation in different types of environmental factors in studies such that results may better be extrapolated to natural amphibian populations. In future studies, the monitoring of stress hormones may also provide

insights into the mechanisms of pesticide's effects on amphibians, which is ultimately crucial to our understanding of neonicotinoid and other pesticides' ecotoxicology.

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Tables and Figures

Table 1. Model outputs from final models of distance travelled (linear model, latency to first movement (proportion; generalized linear model (GLM)) and time spent in the center (proportion; GLM) by wood frog (*Lithobates sylvaticus*) tadpoles at Gosner (1960) stage ~30, 42, and 45/46. Population *Density* (*Dens* when part of an interaction) and *Imidacloprid* concentration (two levels) were z-transformed to be continuous from -0.5 to 0.5 for low/absent and high/present values respectively. Categorical variables included *Arena* (1 to 4), *Wall* (right, left, or no wall), and *Weather* (cloudy (fixed) or sunny), where significances are specified in the footnotes when applicable. SE stands for standard error. Significant effects are in bold.

Stage	Sample Size	Dependent Variables	Independent Variables	Estimate	SE	*t* value	*p* value
~30	280	Distance	**Density**	**0.243**	**0.112**	**2.172**	**0.031**
		P. Latency	***Arena**	**n/a**	**n/a**	**n/a**	**n/a**
			Density	-0.536	0.313	1.715	0.087
		P. Center	**Acclimation**	**0.226**	**0.095**	**2.367**	**0.019**
			Temperature	**-0.219**	**0.103**	**2.129**	**0.034**
42	265	Distance	**Density**	**-0.300**	**0.115**	**2.600**	**0.010**
			Time	**0.169**	**0.058**	**2.924**	**0.004**
		P. Latency	**Density**	**0.869**	**0.314**	**2.765**	**0.006**
			Time	**-0.569**	**0.155**	**3.682**	**< 0.001**
		P. Center	Julian Date	0.454	0.234	1.937	0.054
			Time	0.431	0.252	1.707	0.089
			Temperature	-0.351	0.180	1.954	0.052
45/46	150	Distance	**Mass**	**0.136**	**0.046**	**2.966**	**0.004**
			†Wall	**n/a**	**n/a**	**n/a**	**n/a**
			Julian Date	**-0.148**	**0.060**	**2.479**	**0.014**
			Weather	-0.267	0.145	1.844	0.067
		Latency	**Mass*Density**	**-0.991**	**0.347**	**2.854**	**0.005**
			Mass	**-0.416**	**0.173**	**2.410**	**0.017**
			Weather	**1.380**	**0.356**	**3.874**	**< 0.001**
			‡Wall	**n/a**	**n/a**	**n/a**	**n/a**
			Julian Date	**0.450**	**0.146**	**3.091**	**0.002**
			Imidacloprid	-0.470	0.268	1.758	0.081
			Density	-0.428	0.396	1.080	0.282
		P. Center	**Mass*Density**	**-0.746**	**0.325**	**2.297**	**0.023**
			Mass	**-0.524**	**0.162**	**3.240**	**0.001**
			Julian Date	**0.467**	**0.164**	**2.851**	**0.005**
			§Wall	**n/a**	**n/a**	**n/a**	**n/a**
			Weather	0.716	0.374	1.916	0.057
			Density	-0.206	0.332	0.621	0.535

*Arena: Deviance = 4.125, Df = 3, $p = 0.03$
†Wall: $F_{(2,144)} = 4.528$, $p = 0.012$
‡Wall: Deviance = 2.757, Df = 2, $p = 0.009$
§Wall: Deviance = 3.501, Df = 2, $p = 0.02$

Figure 1. Significant interaction between final mass at metamorphosis and population density for A) latency to first movement, and B) time spent in the center of the arena (both proportional) during open-field tests for wood frogs (*Lithobates sylvaticus*) at Gosner (1960) stages 45/46.

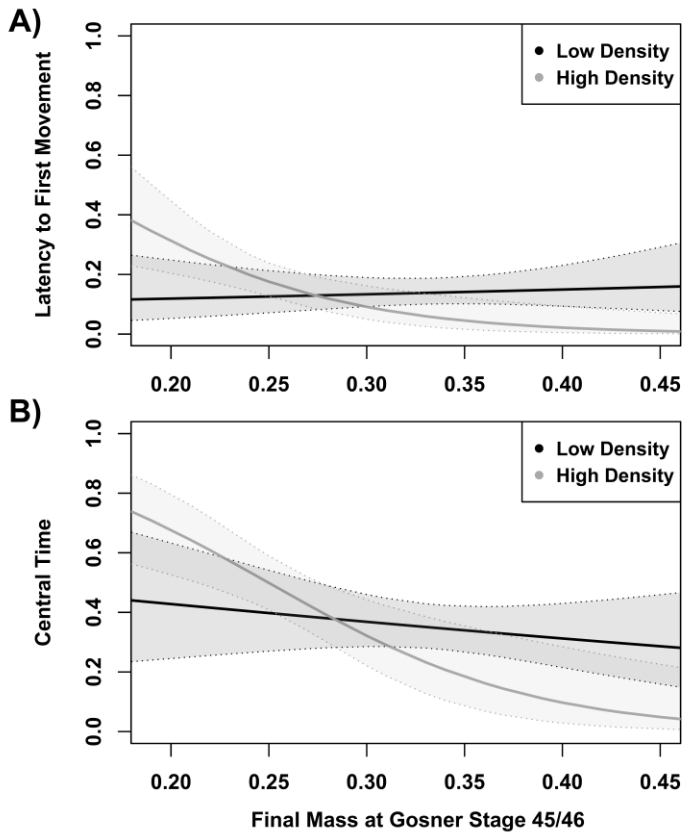
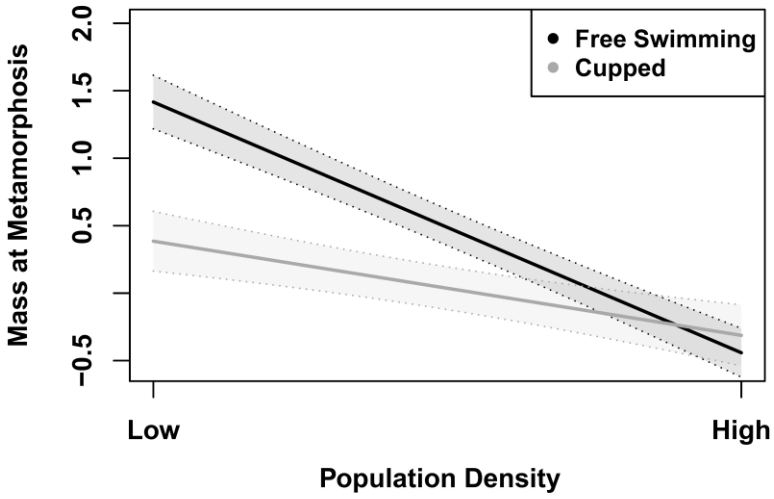


Figure 2. Significant interaction between rearing condition (cupped or free swimming) and population density for final mass at metamorphosis for wood frogs (*Lithobates sylvaticus*) at Gosner (1960) stages 45/46.



Supplementary Materials

Open-field Box

The open field box (*Supplemental Figure 1*) was constructed of ¾ inch plywood and was 25x25x25¾ inches. The walls and ceiling were supported by 1.5x1.5x24¼ inch wooden corner pieces, inset ¾ inch from each side such that walls would be flush with the base of the box. Two opposite walls were hinged and equipped with external handles for transport and to open the box. The box doors were held shut by cabinet magnets and by a sliding lock placed at the top of the door. The floor of the open field box had four silicone semicircles and some markers to ensure the arenas were always in the same position and orientation. A central wooden pole with a whiteboard, the height of which was lower than the arena walls, was mounter in the center of the floor and used to identify which individuals were being tested. On the ceiling of the arena, two COB LED Wall Switch Lights were fixed beside the camera aperture hole and aligned between arena pairs. These provided a steady ambient light for the tadpoles. The ceiling of the open field box had a 2-inch diameter hole cut in the center for the camera aperture. Two screws were also placed on the outside to secure the camera into the same position during every test. A plastic garden pot was also placed on top of the camera after filming began to protect the camera and prevent light from entering through the aperture. Finally, four additional holes were pierced above the center of each arena, where four funnels were securely inserted and used for the addition of predation cues.

Method validation

For method validations see supplementary table S1. Water tests of treated tanks revealed that none of the clean water treatments were contaminated beyond the detection limit of 0.04 µg/L

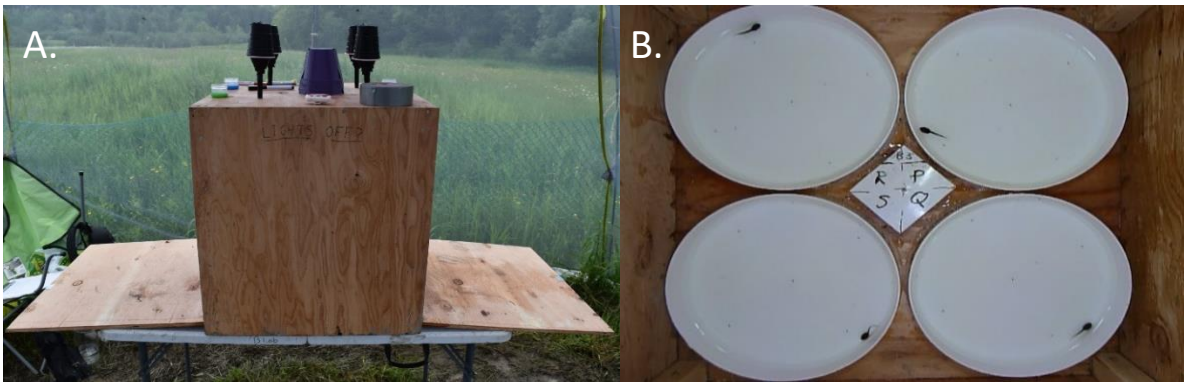
during the experiment. In the inoculated tanks, concentration of imidacloprid averaged 5.78 ± 1.74, 6.39 ± 0.76, and 0.74 ± 0.09 µg/L (range: 3.64-8.76, 5.52-7.3, and 0.65-0.872 µg/L respectively) on May 16 (first application), June 6 (second application) and July 18 (end of experiment), 2019, respectively. Post-hoc ANOVA tests showed that concentration of imidacloprid did not significantly differ between inoculated tanks, or density treatments within these tanks (all $p > 0.05$). Water samples from the egg mass sampling sites and the municipal aqueduct system had trace amounts of imidacloprid, concentrations ranging from 0.001 to 0.004 µg/L (detection limit for these tests of 0.00004 µg/L), despite closest farms not using any neonicotinoids (Canada Department of Agriculture Experimental Farms, *personal communication*). However, these concentrations are many orders of magnitude lower than those found in the tanks, and although tap water was used to fill the tanks, concentrations had reached 0µg/L by the time tadpoles were added based on concentrations of clean water treatment samples which had no detectable traces of imidacloprid in them at that time.

Post-hoc predation cue analyses

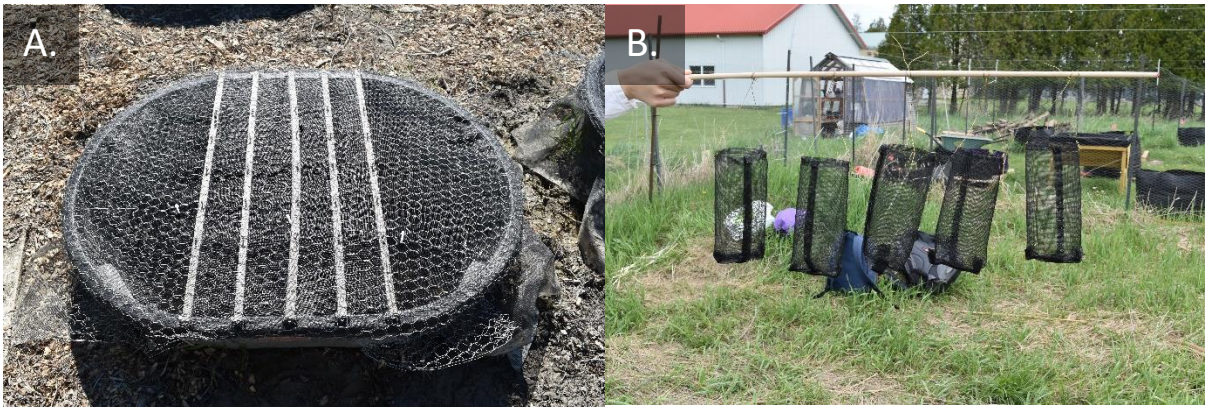
Since I knew from a pilot study that tadpoles did do not alter activity over the first four minutes of testing (accounting for quadratic tendencies, $n = 40$; *unpublished results*), I investigated whether cues were perceived and elicited the expected freeze response. In these post-hoc analyses, pre- and post-cue behaviours were treated as the same behaviour (dependent variable) and fixed effects included those of behavioural analyses, with the addition of predation cue (absent/present; -0.5 and 0.5 respectively) as fixed effect with interactions with treatment variables, up to three way interactions. Tadpole ID was also included as random effect for

distance travelled but not for latency or central time due to their quasi-binomial nature. This revealed that alarm cues lead to significantly lower distances travelled (*cue*: -0.342 ± 0.054, t = 6.368, $p < 0.001$) and greater latencies to first movement (*cue*: 1.37 ± 0.184, t = 7.441, $p <$ 0.001) at Gs 42.

Supplemental Figure 1. A. Open-field box used to house and film B. the four aquatic open-field arenas.



Supplemental Figure 2. A. Mesocosm tank with B. wooden dowels used to hold the cups in in the water column.



Supplemental Table 1. Model outputs for validations that treatments and tanks did not differ in terms of pH (*n* =36), temperature (n = 768; measured daily for each tank) and imidacloprid treatment (in treated tanks; *n* = 18). pH and imidacloprid concentrations were taken from water samples of each tank. Differences between treatments were evaluated using linear models (LM) and the fixed effects were population density (*Dens* when part of an interaction; low/high), imidacloprid presence (*IMI* when part of an interaction; absent/present), and their interaction. These variables were treated as centered continuous variables by assigning a value of -0.5 and 0.5 for each respective level. Analyses of differences between tanks were evaluated separately using ANOVAs (ANOVA outputs can be found below the table). Analyzes of differences in imidacloprid concentrations were performed on the subset of the tanks which had been exposed. All analyses were conducted in R 3.6.3 (R Core Team 2020) and significant effects are in bold.

Dependent Variable	Independent Variable	Estimate	Standard Error	*t* value	*p* value
pH	IMI×Dens	-0.170	0.148	1.147	0.260
	Imidacloprid	0.057	0.074	0.769	0.448
	Density	**0.175**	**0.074**	**2.350**	**0.025**
	*Tank	n/a	n/a	n/a	0.916
Imidacloprd (Treated Tanks)	Density	0.005	0.486	0.01	0.992
	†Tank	n/a	n/a	n/a	0.999
Temperature	IMI×Dens	0.023	0.145	0.162	0.871
	Imidacloprid	0.057	0.072	0.79	0.430
	Density	0.029	0.072	0.40	0.689
	‡Tank	n/a	n/a	n/a	0.999

*Tank = $F_{(11,24)} = 0.805$, $p = 0.635$
†Tank = $F_{(5,12)} = 0.042$, $p = 0.999$
‡Tank = $F_{(11,756)} = 0.150$, $p = 0.999$

Supplemental Table 2. Full model of distance travel at each Gosner stage (Gs; n = 280, 265, and 150 for Gs ~30, 42 and 45/46) evaluated as a linear model (LM) with a gaussian distribution. Estimates and associated *p* values are those obtained before removal of the variable during model selection, or in the final models. Population *Density* (*Dens* when part of an interaction) and *Imidacloprid* concentration (*IMI* when part of an interaction; each with two levels) were z-transformed to be continuous from -0.5 to 0.5 for low/absent and high/present values respectively. Categorical variables included *Arena* (1 to 4), *Wall* (right, left, or no wall), and *Weather* (cloudy (fixed) or sunny), where significances are specified in the footnotes when applicable. SA stands for surface area. Significant effects are in bold.

Gosner Stage	Dependent Variable	Estimate	Standard Error	*t* value	*p* value
~30	IMI×Dens×SA	-0.029	0.263	0.110	0.912
	IMI×Dens	0.425	0.418	1.016	0.311
	IMI×SA	-0.150	0.125	1.203	0.230
	Dens×SA	0.040	0.122	0.329	0.742
	Imidacloprid	0.129	0.113	1.140	0.255
	Density	**0.243**	**0.112**	**2.172**	**0.031**
	Surface Area	-0.012	0.059	0.207	0.836
	Acclimation	-0.092	0.056	1.637	0.103
	Julian Date	0.027	0.091	0.300	0.765
	Time of Day	0.021	0.063	0.337	0.736
	Water Temp.	-0.034	0.157	0.216	0.829
	Weather	0.120	0.132	0.902	0.368
	*Arena	n/a	n/a	n/a	0.579
42	IMI×Dens	-0.110	0.237	0.465	0.643
	Imidacloprid	0.012	0.121	0.103	0.918
	Density	**-0.300**	**0.115**	**2.600**	**0.010**
	Acclimation	0.048	0.057	0.843	0.400
	Julian Date	-0.081	0.090	0.900	0.369
	Time of Day	**0.169**	**0.058**	**2.924**	**0.004**
	Water Temp.	-0.034	0.104	0.329	0.743
	Weather	-0.312	0.227	1.373	0.171
	†Arena	n/a	n/a	n/a	0.864
45/46	IMI×Dens×Mass	-0.378	0.262	1.446	0.151
	IMI×Dens	-0.184	0.192	0.958	0.340
	IMI×Mass	-0.169	0.119	1.421	0.158
	Dens×Mass	0.160	0.122	1.312	0.192
	Imidacloprid	0.118	0.105	1.119	0.265
	Density	0.185	0.119	1.556	0.122
	Mass	**0.136**	**0.046**	**2.966**	**0.004**
	Julian Date	**-0.148**	**0.060**	**2.479**	**0.014**
	Time of Day	0.105	0.150	0.704	0.482
	Air Temp.	-0.056	0.069	0.812	0.419
	Weather	-0.267	0.145	1.844	0.067
	‡Wall/Blind	**n/a**	**n/a**	**n/a**	**0.012**

*Arena: $F_{(3,268)} = 0.657$, $p = 0.579$
†Arena: $F_{(3,254)} = 0.247$, $p = 0.864$
‡Wall: $F_{(2,144)} = 4.528$, $p = 0.012$

Supplemental Table 3. Full model of proportional latency to first movement at each Gosner stage (Gs; n = 280, 265, and 150 for Gs ~30, 42 and 45/46) evaluated as a generalized linear model (GLM) with a quasi-binomial distribution. Estimates and associated *p* values are those obtained before removal of the variable during model selection, or in the final models. Population *Density* (*Dens* when part of an interaction) and *Imidacloprid* concentration (*IMI* when part of an interaction; each with two levels) were z-transformed to be continuous from -0.5 to 0.5 for low/absent and high/present values respectively. Categorical variables included *Arena* (1 to 4), *Wall* (right, left, or no wall), and *Weather* (cloudy (fixed) or sunny), where significances are specified in the footnotes when applicable. SA stands for surface area. Significant effects are in bold.

Gosner Stage	Dependent Variable	Estimate	Standard Error	*t* value	*p* value
~30	IMI×Dens×SA	-1.125	0.717	1.569	0.118
	IMI×Dens	-0.443	1.158	0.382	0.703
	IMI×SA	0.161	0.335	0.481	0.631
	Dens×SA	-0.017	0.351	0.048	0.962
	Imidacloprid	-0.230	0.308	0.747	0.456
	Density	-0.536	0.312	1.715	0.087
	Surface Area	-0.064	0.167	0.387	0.699
	Acclimation	0.215	0.133	1.620	0.106
	Julian Date	0.152	0.170	0.894	0.372
	Time of Day	-0.043	0.372	0.117	0.907
	Water Temp.	0.073	0.225	0.326	0.745
	Weather	-0.136	0.406	0.335	0.738
	***Arena**	**n/a**	**n/a**	**n/a**	**0.030**
42	IMI×Dens	0.643	0.647	0.993	0.322
	Imidacloprid	-0.021	0.320	0.065	0.948
	Density	0.869	0.314	2.765	0.006
	Acclimation	0.142	0.145	0.976	0.330
	Julian Date	-0.293	0.205	1.431	0.154
	Time of Day	-0.569	0.155	3.682	< 0.001
	Water Temp.	0.113	0.240	0.473	0.637
	Weather	0.305	0.557	0.546	0.585
	†Arena	n/a	n/a	n/a	0.586
45/46	IMI×Dens×Mass	0.486	0.731	0.665	0.507
	IMI×Dens	0.399	0.527	0.758	0.450
	IMI×Mass	0.126	0.327	0.384	0.702
	Dens×Mass	**-0.991**	**0.347**	**2.854**	**0.005**
	Imidacloprid	-0.470	0.268	1.758	0.081
	Density	-0.428	0.396	1.080	0.282
	Mass	**-0.416**	**0.173**	**2.410**	**0.017**
	Julian Date	**0.450**	**0.146**	**3.091**	**0.002**
	Time of Day	-0.045	0.359	0.126	0.900
	Air Temp.	0.051	0.201	0.254	0.800
	Weather	**1.380**	**0.356**	**3.874**	**< 0.001**
	‡Wall/Blind	**n/a**	**n/a**	**n/a**	**0.009**

*Arena: Deviance = 4.125, Df = 3, $p = 0.030$
†Arena: Deviance = 1.482, Df = 3, $p = 0.586$
‡Wall: Deviance = 2.757, Df = 2, $p = 0.009$

Supplemental Table 4. Full model of proportional time spent in the center of the arena at each Gosner stage (Gs; n = 280, 265, and 150 for Gs ~30, 42 and 45/46) evaluated as a generalized linear model (GLM) with a quasi-binomial distribution. Estimates and associated *p* values are those obtained before removal of the variable during model selection, or in the final models. Population *Density* (*Dens* when part of an interaction) and *Imidacloprid* concentration (*IMI* when part of an interaction; each with two levels) were z-transformed to be continuous from -0.5 to 0.5 for low/absent and high/present values respectively. Categorical variables included *Arena* (1 to 4), *Wall* (right, left, or no wall), and *Weather* (cloudy (fixed) or sunny), where significances are specified in the footnotes when applicable. SA stands for surface area. Significant effects are in bold.

Gosner Stage	Dependent Variable	Estimate	Standard Error	*t* value	*p* value
~30	IMI×Dens×SA	0.028	0.448	0.062	0.951
	IMI×Dens	-0.863	0.709	1.217	0.225
	IMI×SA	0.266	0.216	1.233	0.219
	Dens×SA	0.361	0.204	1.774	0.077
	Imidacloprid	0.157	0.196	0.802	0.423
	Density	-0.072	0.197	0.366	0.715
	Surface Area	0.141	0.096	1.476	0.141
	Acclimation	**0.226**	**0.095**	**2.367**	**0.019**
	Julian Date	0.009	0.155	0.057	0.955
	Time of Day	0.043	0.206	0.209	0.835
	Water Temp.	**-0.219**	**0.103**	**2.129**	**0.034**
	Weather	-0.200	0.291	0.689	0.491
	*Arena	n/a	n/a	n/a	0.691
42	IMI×Dens	-0.769	0.510	1.509	0.133
	Imidacloprid	-0.244	0.256	0.954	0.341
	Density	-0.006	0.262	0.021	0.983
	Acclimation	-0.037	0.130	0.285	0.776
	Julian Date	0.454	0.234	1.937	0.054
	Time of Day	0.431	0.252	1.707	0.089
	Water Temp.	-0.351	0.180	1.954	0.052
	Weather	-0.217	0.514	0.423	0.673
	†Arena	n/a	n/a	n/a	0.917
45/46	IMI×Dens×Mass	0.530	0.719	0.737	0.462
	IMI×Dens	0.532	0.504	1.056	0.293
	IMI×Mass	-0.147	0.315	0.465	0.642
	Dens×Mass	**-0.746**	**0.325**	**2.297**	**0.023**
	Imidacloprid	-0.118	0.251	0.472	0.638
	Density	-0.206	0.332	0.621	0.535
	Mass	**-0.524**	**0.162**	**3.240**	**0.001**
	Julian Date	**0.467**	**0.164**	**2.851**	**0.005**
	Time of Day	-0.188	0.385	0.487	0.627
	Air Temp.	0.126	0.200	0.632	0.528
	Weather	0.716	0.374	1.916	0.057
	‡Wall/Blind	**n/a**	**n/a**	**n/a**	**0.020**

*Arena: Deviance = 0.475, Df = 3, $p = 0.691$
†Arena: Deviance = 0.166, Df = 3, $p = 0.917$
‡Wall: Deviance = 3.501, Df = 2, $p = 0.020$

Supplemental Table 5. Full model of distance travelled in the presence of alarm cues at each Gosner stage (Gs; n = 280 and 265 for Gs ~30 and 42) evaluated as a linear model (LM) with a gaussian distribution. Estimates and associated *p* values are those obtained before removal of the variable during model selection, or in the final models. Population *Density* (*Dens* when part of an interaction) and *Imidacloprid* concentration (*IMI* when part of an interaction; each with two levels) were z-transformed to be continuous from -0.5 to 0.5 for low/absent and high/present values respectively. Categorical variables included *Arena* (1 to 4), *Wall* (right, left, or no wall), and *Weather* (cloudy (fixed) or sunny), where significances are specified in the footnotes when applicable. SA stands for surface area. Significant effects are in bold.

Gosner Stage	Dependent Variable	Estimate	Standard Error	*t* value	*p* value
~30	IMI×Dens×SA	-0.064	0.225	0.286	0.775
	IMI×Dens	-0.151	0.360	0.419	0.675
	IMI×SA	0.045	0.108	0.415	0.678
	Dens×SA	-0.066	0.103	0.638	0.524
	Imidacloprid	**0.277**	**0.100**	**2.782**	**0.006**
	Density	**0.201**	**0.100**	**2.016**	**0.045**
	Pre-cue Distance	**0.579**	**0.049**	**11.734**	**< 0.001**
	Surface Area	0.092	0.050	1.862	0.064
	Acclimation	-0.070	0.053	1.304	0.193
	Julian Date	0.095	0.066	1.432	0.153
	Time of Day	0.146	0.098	1.489	0.138
	Water Temp.	-0.098	0.051	1.919	0.056
	Weather	-0.061	0.189	0.324	0.746
	*Arena	n/a	n/a	n/a	0.948
42	IMI×Dens	0.182	0.198	0.920	0.359
	Imidacloprid	0.035	0.100	0.351	0.726
	Density	0.053	0.099	0.541	0.589
	Pre-cue Distance	**0.660**	**0.049**	**13.464**	**< 0.001**
	Acclimation	0.035	0.048	0.729	0.466
	Julian Date	0.019	0.078	0.241	0.810
	Time of Day	0.015	0.096	0.155	0.878
	Water Temp.	**-0.171**	**0.048**	**3.557**	**< 0.001**
	Weather	-0.195	0.167	1.171	0.243
	†Arena	n/a	n/a	n/a	0.732

*Arena: $F_{(3,258)} = 0.120$, $p = 0.948$
†Arena: $F_{(3,249)} = 0.430$, $p = 0.732$

Supplemental Table 6. Full model of proportional latency to first movement in the presence of alarm cues at each Gosner stage (Gs; n = 280 and 265 for Gs ~30 and 42) evaluated as a generalized linear model (GLM) with a quasi-binomial distribution. Estimates and associated *p* values are those obtained before removal of the variable during model selection, or in the final models. Population *Density* (*Dens* when part of an interaction) and *Imidacloprid* concentration (*IMI* when part of an interaction; each with two levels) were z-transformed to be continuous from -0.5 to 0.5 for low/absent and high/present values respectively. P. P. Latency stands for pre-alarm-cue proportional latency to first movement. Categorical variables included *Arena* (1 to 4), *Wall* (right, left, or no wall), and *Weather* (cloudy (fixed) or sunny), where significances are specified in the footnotes when applicable. SA stands for surface area. Significant effects are in bold.

Gosner Stage	Dependent Variable	Estimate	Standard Error	*t* value	*p* value
~30	IMI×Dens×SA	0.493	0.498	0.989	0.324
	IMI×Dens	-0.586	0.777	0.754	0.452
	IMI×SA	-0.005	0.235	0.023	0.981
	Dens×SA	-0.570	0.779	0.731	0.465
	Imidacloprid	-0.117	0.219	0.536	0.593
	Density	-0.203	0.242	0.841	0.401
	P. P. Latency	0.411	0.504	0.816	0.415
	Surface Area	0.015	0.116	0.128	0.899
	Acclimation	0.088	0.099	0.881	0.379
	Julian Date	**0.267**	**0.107**	**2.506**	**0.013**
	Time of Day	-0.035	0.117	0.303	0.762
	Water Temp.	0.166	0.252	0.658	0.511
	Weather	0.085	0.392	0.217	0.828
	*Arena	n/a	n/a	n/a	0.935
42	IMI×Dens	-0.020	0.470	0.043	0.966
	Imidacloprid	-0.002	0.239	0.010	0.992
	Density	0.109	0.235	0.463	0.644
	P. P. Latency	**1.913**	**0.379**	**5.049**	**< 0.001**
	Acclimation	-0.219	0.115	1.903	0.058
	Julian Date	0.248	0.172	1.445	0.150
	Time of Day	-0.033	0.253	0.132	0.895
	Water Temp.	0.222	0.118	1.882	0.061
	Weather	**0.879**	**0.438**	**2.009**	**0.046**
	†Arena	n/a	n/a	n/a	0.520

*Arena: Deviance = 0.203, Df = 3, $p = 0.935$
†Arena: Deviance = 1.664, Df = 3, $p = 0.520$

Supplemental Table 7. Full model of proportional time spent in the center of the arena in the presence of alarm cues at each Gosner stage (Gs; n = 280 and 265 for Gs ~30 and 42) evaluated as a generalized linear model (GLM) with a quasi-binomial distribution. Estimates and associated *p* values are those obtained before removal of the variable during model selection, or in the final models. Population *Density* (*Dens* when part of an interaction) and *Imidacloprid* concentration (*IMI* when part of an interaction; each with two levels) were z-transformed to be continuous from -0.5 to 0.5 for low/absent and high/present values respectively. P. P. Center stands for pre-alarm-cue proportional central time to first movement. Categorical variables included *Arena* (1 to 4), *Wall* (right, left, or no wall), and *Weather* (cloudy (fixed) or sunny), where significances are specified in the footnotes when applicable. SA stands for surface area. Significant effects are in bold.

Gosner Stage	Dependent Variable	Estimate	Standard Error	*t* value	*p* value
~30	IMI×Dens×SA	0.136	0.402	0.339	0.735
	IMI×Dens	-0.196	0.644	0.305	0.761
	IMI×SA	0.225	0.193	1.169	0.244
	Dens×SA	0.193	0.189	1.020	0.309
	Imidacloprid	-0.174	0.180	0.969	0.334
	Density	0.059	0.184	0.321	0.748
	P. P. Center	**1.408**	**0.370**	**3.805**	**< 0.001**
	Surface Area	0.022	0.096	0.232	0.817
	Acclimation	0.084	0.091	0.923	0.357
	Julian Date	0.049	0.140	0.349	0.727
	Time of Day	-0.310	0.190	1.633	0.104
	Water Temp.	0.045	0.090	0.502	0.616
	Weather	-0.359	0.278	1.290	0.198
	*Arena	n/a	n/a	n/a	0.168
42	IMI×Dens	0.542	0.666	0.813	0.420
	Imidacloprid	-0.334	0.328	1.020	0.309
	Density	-0.281	0.319	0.882	0.379
	P. P. Center	**2.944**	**0.671**	**4.389**	**< 0.001**
	Acclimation	0.095	0.153	0.622	0.534
	Julian Date	0.290	0.287	1.009	0.314
	Time of Day	**0.343**	**0.158**	**2.175**	**0.031**
	Water Temp.	-0.089	0.297	0.298	0.766
	Weather	0.775	0.895	0.866	0.387
	†Arena	n/a	n/a	n/a	0.207

*Arena: Deviance = 1.353, Df = 3, $p = 0.168$
†Arena: Deviance = 2.367, Df = 3, $p = 0.207$

Supplemental Table 8. Full models of final mass at metamorphosis (linear mixed model with tank ID as random effect; n = 989) and time to metamorphosis (generalized linear model with a quasi-Poisson distribution; n = 994). Estimates and associated *p* values are those obtained before removal of the variable during model selection, or in the final models. Population *Density* (*Dens* when part of an interaction), *Imidacloprid* concentration (*IMI* when part of an interaction), and rearing condition (Cup) were z-transformed to be continuous from -0.5 to 0.5 for low/absent, high/present, and free/cupped values respectively. Significant effects are in bold.

Independent Variable	Dependent Variable	Estimate	Standard Error	*t* value	*p* value
Mass	IMI×Dens×Cup	-0.060	0.235	0.253	0.800
	IMI×Dens	0.121	0.255	0.478	0.645
	IMI×Cup	-0.082	0.116	0.709	0.478
	Dens×Cup	**1.160**	**0.117**	**9.901**	**< 0.001**
	Imidacloprid	0.209	0.121	1.720	0.120
	Density	**-1.277**	**0.137**	**9.284**	**< 0.001**
	Cup	**-0.451**	**0.117**	**9.901**	**< 0.001**
Metamorphosis Time	IMI×Dens×Cup	0.021	0.016	1.314	0.189
	IMI×Dens	-0.007	0.006	1.044	0.297
	IMI×Cup	-0.006	0.008	0.756	0.450
	Dens×Cup	-0.005	0.008	0.650	0.516
	Imidacloprid	**-0.009**	**0.003**	**3.343**	**0.001**
	Density	**-0.020**	**0.003**	**6.014**	**< 0.001**
	Cup	**-0.014**	**0.004**	**3.546**	**< 0.001**

Supplemental Table 9. Repeatabilities of distance travelled by wood frogs (*Lithobates sylvaticus*) during open-field tests separated according to developmental stage (Gosner 1960, stages ~30, 42 and 45/46) and experimental treatments (Density: Low and High; Imidacloprid: Absent and Present). *Both* indicates that both treatments are considered in the analyses. Significant effects are in bold.

Stage	Density	Imidacloprid	Repeatability	SE	Confidence Interval	*p* (LRT)	*p* (Permutation)
30 & 42	**Both**	**Both**	**0.150**	**0.061**	**0.043 – 0.284**	**0.012**	**0.029**
	Low	**Both**	**0.209**	**0.084**	**0.063 – 0.399**	**0.013**	**0.027**
	High	Both	0.138	0.088	0.000 – 0.342	0.072	0.104
	Both	Absent	0.099	0.085	0.000 – 0.312	0.145	0.246
	Both	Present	0.122	0.087	0.000 – 0.327	0.102	0.177
	Low	Absent	0.152	0.120	0.000 – 0.442	0.117	0.238
	High	Absent	0.073	0.113	0.000 – 0.390	0.294	0.508
	Low	Present	0.135	0.122	0.000 – 0.441	0.162	0.303
	High	Present	0.158	0.116	0.000 – 0.447	0.107	0.218
42 & 45/46	Both	Both	0.028	0.068	0.000 – 0.217	0.458	0.490
	Low	Both	0.086	0.096	0.000 – 0.326	0.319	0.323
	High	Both	0	0.089	0.000 – 0.288	1	1
	Both	Absent	0	0	0.000 – 0.000	1	0.655
	Both	Present	0.082	0.100	0.000 – 0.347	0.331	0.323
	Low	Absent	0	0.109	0.000 – 0.352	1	1
	High	Absent	0.014	0.150	0.000 – 0.494	1	0.615
	Low	Present	0.268	0.146	0.000 – 0.582	0.128	0.107
	High	Present	0	0.111	0.000 – 0.371	1	1
30 & 45/46	Both	Both	0	0.056	0.000 – 0.185	1	1
	Low	Both	0	0.078	0.000 – 0.250	1	1
	High	Both	0	0.08	0.000 – 0.261	1	1
	Both	Absent	0	0.083	0.000 – 0.271	1	1
	Both	Present	0	0.079	0.000 – 0.257	1	1
	Low	Absent	0	0.113	0.000 – 0.370	1	1
	High	Absent	0	0.137	0.000 – 0.454	1	1
	Low	Present	0	0.122	0.000 – 0.392	1	1
	High	Present	0	0.122	0.000 – 0.393	1	1
30, 42 & 45/46	**Both**	**Both**	**0.092**	**0.045**	**0.010 – 0.187**	**0.039**	**0.021**
	Low	**Both**	**0.134**	**0.064**	**0.027 – 0.277**	**0.029**	**0.028**
	High	Both	0.069	0.057	0.000 – 0.202	0.184	0.160
	Both	Absent	0.029	0.050	0.000 – 0.168	0.358	0.392
	Both	Present	0.109	0.062	0.000 – 0.243	0.070	0.054
	Low	Absent	0.058	0.076	0.000 – 0.247	0.271	0.279
	High	Absent	0.010	0.069	0.000 – 0.233	0.474	0.506
	Low	Present	0.147	0.088	0.000 – 0.336	0.074	0.072
	High	Present	0.091	0.080	0.000 – 0.277	0.192	0.195

Supplemental Table 10. Repeatabilities of distances travelled by wood frog tadpoles before and after the addition of predation cues within the same open-field tests (Gosner stage) and distance travelled in the presence of predation cues between stages (accounting for distance travelled before the addition of the cue). Significant repeatabilities are in bold.

Stage	Density	Imidacloprid	Repeatability	SE	Confidence Interval	*p* (LRT)	*p* (Permutation)
Within Stage 30	**Both**	**Both**	**0.720**	**0.056**	**0.615 – 0.835**	**< 0.001**	**0.001**
	Low	**Both**	**0.564**	**0.057**	**0.467 – 0.685**	**< 0.001**	**0.001**
	High	**Both**	**0.592**	**0.053**	**0.499 – 0.706**	**< 0.001**	**0.001**
	Both	**Absent**	**0.561**	**0.057**	**0.467 – 0.681**	**< 0.001**	**0.001**
	Both	**Present**	**0.579**	**0.057**	**0.486 – 0.706**	**< 0.001**	**0.001**
	Low	**Absent**	**0.591**	**0.077**	**0.451 – 0.752**	**< 0.001**	**0.001**
	High	**Absent**	**0.450**	**0.096**	**0.286 – 0.654**	**< 0.001**	**0.002**
	Low	**Present**	**0.452**	**0.097**	**0.299 – 0.673**	**0.001**	**0.001**
	High	**Present**	**0.720**	**0.056**	**0.615 – 0.835**	**< 0.001**	**0.001**
Within Stage 42	**Both**	**Both**	**0.655**	**0.068**	**0.535 – 0.793**	**< 0.001**	**0.001**
	Low	**Both**	**0.540**	**0.064**	**0.422 – 0.677**	**< 0.001**	**0.001**
	High	**Both**	**0.641**	**0.054**	**0.538 – 0.750**	**< 0.001**	**0.001**
	Both	**Absent**	**0.522**	**0.064**	**0.405 – 0.655**	**< 0.001**	**0.001**
	Both	**Present**	**0.628**	**0.051**	**0.541 – 0.738**	**< 0.001**	**0.001**
	Low	**Absent**	**0.489**	**0.093**	**0.310 – 0.683**	**< 0.001**	**0.001**
	High	**Absent**	**0.604**	**0.081**	**0.466 – 0.771**	**< 0.001**	**0.001**
	Low	**Present**	**0.602**	**0.077**	**0.477 – 0.769**	**< 0.001**	**0.001**
	High	**Present**	**0.655**	**0.068**	**0.535 – 0.793**	**< 0.001**	**0.001**
Predator Stage 30 & 42 (with pre-cue distance as fixed effect)	Both	Both	0.040	0.055	0.000 – 0.186	0.312	0.415
	Low	Both	0.060	0.082	0.000 – 0.281	0.282	0.433
	High	Both	0	0.064	0.000 – 0.209	1	1
	Both	Absent	0.141	0.088	0.004 – 0.344	0.070	0.133
	Both	Present	0	0.068	0.000 – 0.218	1	1
	Low	Absent	0.119	0.119	0.000 – 0.433	0.166	0.384
	High	Absent	0.220	0.125	0.027 – 0.527	0.058	0.154
	Low	Present	0.082	0.13	0.000 – 0.455	0.308	0.515
	High	Present	0	0.099	0.000 – 0.329	1	1

Supplementary Table 11. Final model estimates of the effect and significance of the addition of the predation cue for each measured behaviour at each developmental (Gosner 1960) stage. A linear mixed model (LMM) was used for distance travelled (scaled and square-rooted; tadpole ID as random effect), while a generalized linear model (GLM) with a quasi-binomial distribution were used for proportional latency to first movement and central time (each behaviour was divided by the duration of each trial; 120s). Fixed effects include those specified in materials and methods. Significant effects are in bold.

Gosner Stage	Behaviour	Estimate	Standard Error	*t* value	*p* value
~30	**Distance**	**-0.194**	**0.052**	**3.739**	**< 0.001**
	Latency	**1.125**	**0.186**	**6.402**	**< 0.001**
	Central Time	0.047	0.129	0.367	0.714
42	**Distance**	**-0.342**	**0.054**	**6.368**	**< 0.001**
	Latency	**1.370**	**0.184**	**7.441**	**< 0.001**
	Central Time	0.062	0.195	0.317	0.751