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Developmental Toxicity of a Neonicotinoid Insecticide, Acetamiprid to Zebrafish Embryos

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Supporting Information

ABSTRACT: Agricultural use of neonicotinoid insecticides is increasing worldwide, posing a risk to nontarget organisms. The present study investigated developmental toxicity of a widely used neonicotinoid, acetamiprid, to zebrafish embryos. Sublethal (malformations, hatchability, heart rate, body length, alteration of spontaneous movement and touch responses) and lethal effects were monitored during exposure period from 6 h post fertilization (hpf) to 120 hpf. Zebrafish embryos exhibited significant mortality and teratogenic effects at acetamiprid concentration greater than 263 mg/L, with bent spine being the main malformation. Toxicity spectra were constructed to rank the sensitivity of individual end points to acetamiprid exposure and impaired spontaneous movement was the most sensitive end point of those tested. The present study provides the basis for understanding developmental toxicity of acetamiprid exposure to zebrafish embryos. This information is critical for future studies evaluating aquatic risk from neonicotinoids as little is known regarding adverse effects of neonicotinoids to aquatic vertebrate species.

KEYWORDS: *acetamiprid, neonicotinoid insecticides, developmental toxicity, zebrafish embryos, toxicity spectrum*

INTRODUCTION

Neonicotinoid insecticides have been registered in more than 120 countries since their initial arrival on the market in the early 1990s. Since the debut of neonicotinoids, the use of these insecticides has surged, accounting for over 25% of the global insecticide market, as legacy organochlorine and organophosphate insecticides have been gradually phased out worldwide.[1] The reason for their popularity is based on the notion that neonicotinoids act as nicotinic acetylcholine receptor (nAChR) agonists, which are believed to have little effect on vertebrate species and have little cross-resistance with other insecticides.[2] Additionally, neonicotinoids possess systemic activity and as such would distribute throughout the plants more effectively than traditional insecticides to combat target pest species.[3] Nevertheless, the high water solubility of neonicotinoids also causes these compounds to be highly mobile and as a consequence, easily transported into aquatic ecosystems, posing potential risks to nontarget aquatic organisms.[4]

Seven neonicotinoid insecticides are currently used for agricultural production, including acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam.[5] One country where neonicotinoids are heavily used is China, which is the main producer, consumer, and exporter of these compounds. Outside of imidacloprid, acetamiprid has been the most extensively used neonicotinoid in China, with its annual output being nearly 8000 tons.[6] Previous studies indicated that detection frequencies and concentrations of acetamiprid residues in surface water in China were comparable to imidacloprid.[7−9] Although high volumes of acetamiprid are being used and the overall amount being used is still increasing, very limited information regarding the toxicity of acetamiprid to nontarget organisms is available. To date, most toxicity data regarding neonicotinoids are limited to imidacloprid and clothianidin,[4] which hinders the assessment of ecological risk associated with other neonicotinoids such as acetamiprid.

One model species which has been used not only to assess effects on aquatic biota (especially vertebrate ones) but also to bridge the gap to other vertebrates more difficult to study (such as humans) is zebrafish.[10] As an alternative testing tool, fish embryos are becoming more commonly used as they are quite easy to work with due to their small size, transparent body and short developmental cycle.[11] In addition, many studies have shown that adverse outcomes on zebrafish can be extrapolated to mammalian species, such as humans and rats.[12] Therefore, evaluating toxicity of neonicotinoids to zebrafish embryos not only provides information on aquatic toxicity of these insecticides but also sheds a light on their potential impacts to human health and other vertebrate species.

The objective of the current study was to determine the developmental toxicity to zebrafish embryos by acetamiprid exposure. Toxicity end points included mortality, malformations, hatching ability, heart rate, body length, spontaneous movement, and touch response. By doing so, a toxicity spectrum of this compound, which shows the relative sensitivity of each of the tested end points, was established. The use of this baseline information for acetamiprid is highly needed for future chronic toxicity testing using zebrafish to

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better understand the risk of neonicotinoids to nontarget vertebrate species and humans.

MATERIALS AND METHODS

Fish Maintenance and Embryo Collection. Adult wild-type (AB) zebrafish (*Danio rerio*) were obtained from the China Zebrafish Resource Center (Wuhan, China). All fish were reared in a recirculation system at 28 °C and under a 14:10 h light/dark photoperiod following standard zebrafish breeding protocols.[13] Meanwhile, $NaHCO_3$ and NaCl were added to reverse osmosis filtered water to maintain pH and conductivity at 7.0−7.5 and 500−550 μS/cm, respectively. Cultured adult zebrafish were fed twice a day with live artemia (Fengnian Aquaculture Corporation, Tianjin, China). Zebrafish embryos were obtained from paired adult fish in spawning boxes overnight with a female/male ratio of 1:1. Spawning was induced at the beginning of the light cycle (8 am), and within 0.5 h of spawning, embryos were collected, rinsed, and transferred to embryo medium (EM) before use.[13] Embryos, which were fertilized and appeared healthy, were selected after being staged under a stereomicroscope SZX 7 (Olympus, Tokyo, Japan) as described by Kimmel et al.[11]

Chemicals and Reagents. Neat compound of acetamiprid (98.1% purity) was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetamiprid-d_3 and thiamethoxam-d_3 with purities greater than 98% were purchased from CDN Isotopes (Quebec, Canada) and used as surrogate and internal standards, respectively, when analyzing acetamiprid in the EM. Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Protease E (Sigma-Aldrich Corporation, St. Louis, U.S.A.) was used for chorion digestion.

Chemical Analysis. To determine stability of acetamiprid concentrations in EM throughout the bioassays, the samples were collected at the initiation (0 h) and conclusion of the bioassays (120 h). After adding the surrogate, EM samples were diluted to appropriate concentrations with acetonitrile (500−9000 times). After adding the internal standard, acetamiprid was analyzed using a LC-30-AD UHPLC (Shimadzu, Japan) coupled with QTRAP 5500 MS/MS (AB Sciex, U.S.A.) following a previously established method.[7] Average acetamiprid concentrations were within 5.2%−8.6% of nominal concentrations throughout the entirety of all bioassays (Table S1, "S" represents figures and tables in the Supporting Information). All analyses were conducted in three replicates, and measured acetamiprid concentrations were used for all calculations.

Bioassays with Zebrafish. In the present study, mortality, malformations, hatchability, heart rate, body length, spontaneous movement, and touch response were used to assess developmental toxicity of zebrafish embryos after acetamiprid exposure. A solution of acetamiprid (900 mg/L) was prepared in EM, and additional test solutions were prepared using serial dilutions. In total, five sets of bioassays were conducted using various clutches of collected embryos to assess toxicity to zebrafish embryos evaluating a suite of end points under different timeframes (Table S2).

A single clutch (clutch 1) was used to assess mortality, malformations, and hatchability. This bioassay utilized 36 embryos per replicate with three replicates per treatment, resulting in a total of 108 embryos per treatment (i.e., a testing concentration at a given time point). These bioassays were conducted in 96-well plates using 200 μL of solution per well. Viable embryos at 6 h post fertilization (hpf) were individually exposed to acetamiprid in each well. Alternatively, the remaining four bioassays (clutches 2−5) were carried out in 6-well plates with 5 mL of solution in each well. These tests were conducted in triplicate with 20 embryos per replicate for a total of 60 embryos per treatment. All 96-well and 6-well plates were covered with parafilm to prevent evaporation and placed in a light-controlled incubator at 28 ± 0.5 °C with a light/dark cycle of 14:10. Acetamiprid solutions were not renewed during the 120 h bioassays, as its concentrations in EM remained stable throughout the experiments (Table S1). Organisms were staged and evaluated using a charge-coupled device (CCD) camera (Toupcam, China) on the stereomicroscope after exposure, and organisms were allowed to acclimate for 5 min at 27−28 °C before analysis. More details of the five bioassays are discussed below.

Clutch 1. Embryo mortality and malformation were measured after 120 hpf,[14−16] while hatchability was measured at 72 hpf using the stereomicroscope.[11] The bioassay was conducted using 10 concentrations of acetamiprid (54, 107, 263, 374, 433, 537, 644, 760, 848, and 974 mg/L), and an EM only solution was used as the control. Embryo mortality was assessed by counting the number of individuals that lacked heart function. Malformations in embryos were assessed by documenting the teratology daily, starting at 24 hpf and concluding at 120 hpf. The end points of teratology included bent spine, uninflated swim bladder, pericardial edema, yolk sac edema, and malformed tail. Hatchability was recorded as the individuals that were able to rupture the chorion pre-72 hpf.

Clutch 2. Embryo heart rate was measured at 48, 60, and 72 hpf[16,17]after exposure to acetamiprid at three concentrations of 107, 537, and 760 mg/L, and an EM only solution was used as the control. Videos were recorded for 10 s for each embryo using the CCD camera, and the number of beats were calculated as beats per min.

Clutch 3. Growth of zebrafish was evaluated after exposure to acetamiprid concentrations at 54, 107, 263, 374, and 433 mg/L. After 120 hpf, fish larvae were collected, positioned on microscope slides, and photographed using the CCD camera. Body length measurements were made using the Toup View software associated with the camera. The length of individual larvae was measured from the head to the tail (tail fin excluded) based on previous work.[14]

Clutch 4. The impact of acetamiprid on embryo behaviors was analyzed using clutches 4 and 5. Spontaneous movement (alternating tail bending or coiling) of the embryos was assessed hourly between 17 and 27 hpf.[18,19] The tests were conducted using four concentrations of acetamiprid at 107, 537, 760, and 974 mg/L. In order to determine impaired spontaneous movement, the number of independent embryo tail swings per min was recorded using the CCD camera. The counting time from the first to the last well for a single 6-well plate was less than 8 min.

Clutch 5. Touch response was evaluated after 27, 36, and 48 hpf.[20,21] Similar to the spontaneous movement bioassay, four concentrations of acetamiprid (107, 537, 760, and 974 mg/L) and a control were tested. Previous work showed that chorion did not affect the bioavailability, bioaccumulation, and toxicity of hydrophilic compounds such as acetamiprid.[22] As such, chorion of the tested individual was removed before evaluation to accurately assess touch response. To remove the chorion, embryos were digested with 0.1 mg/mL of protease E for 7−10 min on an oscillating table as described by Chen et al.[21] After digestion, the medium was replaced with the EM and the embryos were washed repeatedly using the EM until the chorion was completely removed. Fish response was observed under the stereomicroscope, and the touch response was evoked when the dorsal tail and head regions were touched using an eyelash probe. Tail and head responses were recorded individually for each organism. If body bending or swimming behavior occurred after the initial touch, it was considered a positive response.

Data Analysis. Concentration−response curves were generated using a sigmoidal regression to calculate the 5% and median effect concentrations (EC5 and EC50, respectively) for malformations, hatchability, heart rate, body length, spontaneous movement, and touch response as well as the 5% and median lethal concentrations (LC5 and LC50, respectively) for mortality using Prism 5.0. The teratogenic index (TI) was determined using the generated dose−response curves for mortality and malformations, defined as a ratio of LC50/EC50.[23] Statistical significance between the treatments and controls was determined using a one-way analysis of variance (ANOVA) followed by a posthoc Dunnett's test. A $p < 0.05$ was regarded as a significant difference. All statistical analyses were conducted using SPSS 16.0. All results are shown as mean ± standard error of mean (SEM).

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

RESULTS

Physical Effects. Dose−response curves for embryo mortality, malformations, and impaired hatchability after exposure to acetamiprid are shown in Figure S1. Zebrafish embryos started to exhibit significant mortality (120 hpf) at a concentration of 374 mg/L when compared with controls ($p < 0.05$), with complete mortality observed at 760 mg/L (Figure S1). The LC50 value of acetamiprid at 120 hpf for zebrafish embryos was 518 (469−572) mg/L (Table S2). Acetamiprid also caused various defects in embryos, including bent spine, uninflated swim bladder, pericardial edema, and yolk sac edema, with bent spine being the most frequently observed malformation (Figure 1). The EC50 for malformations at 120 hpf was 323 (303−344) mg/L, and significant differences between the treatments and controls were observed as low as 263 mg/L, with all individuals showing deformities at 760 mg/L (Figure S1 and Table S2). Accordingly, the TI (LC50/EC50) was calculated to be 1.6 for all malformations. Hatchability was only significantly decreased at concentrations greater than 537 mg/L (Figure S1), and impaired hatchability had a 72 hpf EC50 of 554 (485−633) mg/L (Table S2).

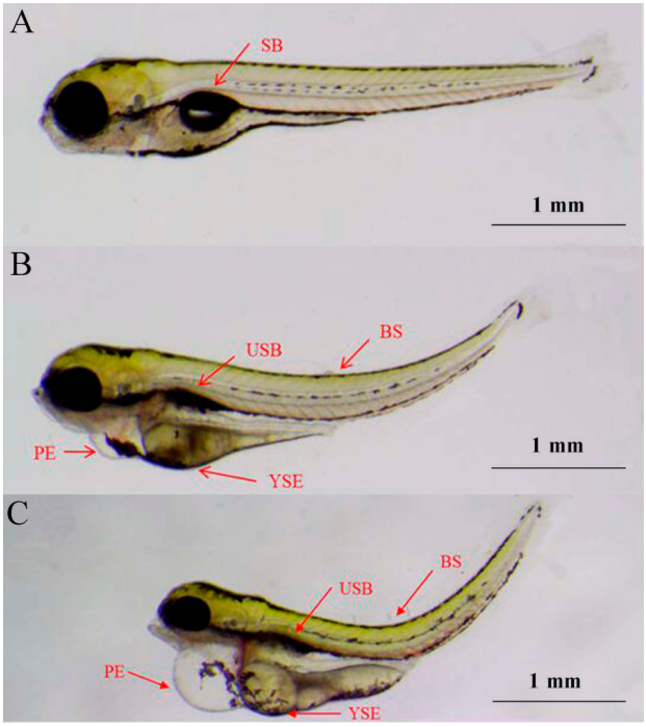

Figure 1. Visual images of zebrafish embryos at 120 hpf in control (A) and acetamiprid exposures (B, 263 mg/L; C, 644 mg/L). SB, swim bladder; USB, uninflated swim bladder; PE, pericardial edema; YSE, yolk sac edema; BS, bent spine.

In the heart rate bioassay (clutch 2), survival was greater than 80% for all treatments and deceased individuals were not included in the analysis. Exposure to acetamiprid significantly reduced heart rates for zebrafish embryos at 48, 60, and 72 hpf for all treatments (≥107 mg/L of acetamiprid) when compared to the control, with the exception of the 72 hpf embryos at 107 mg/L (Figure 2). The degree of difference between the treatments and the control was similar regardless of exposure concentrations tested or time periods (typically decreasing between 20% and 35% with few exceptions). As no reduced heart rates were higher than 50% in any treatments, an EC50 value could not be determined.

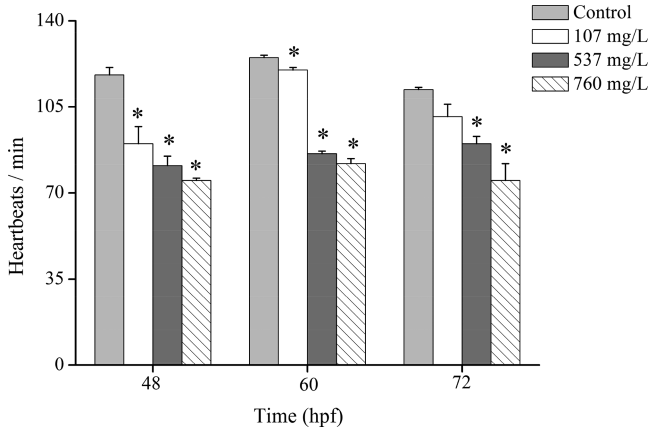

Figure 2. Heartbeat analysis for zebrafish embryos (beats/min, mean ± SEM, $n = 3$) at 48, 60, and 72 hpf after exposure to four acetamiprid concentrations (0, 107, 537, and 760 mg/L). An asterisk (*) signifies a significant difference ($p < 0.05$) compared with control.

In the body length bioassay (clutch 3), deceased individuals were excluded from the analysis. Body length of larval fish followed a dose−response relationship similar to other end points tested (Figure 3). In all acetamiprid exposure concentrations tested (≥54 mg/L), significant differences from the control were noted. Similar to the heart rate analysis, reduced growth was less than 50% even at the highest concentration, thus no EC50 was derived.

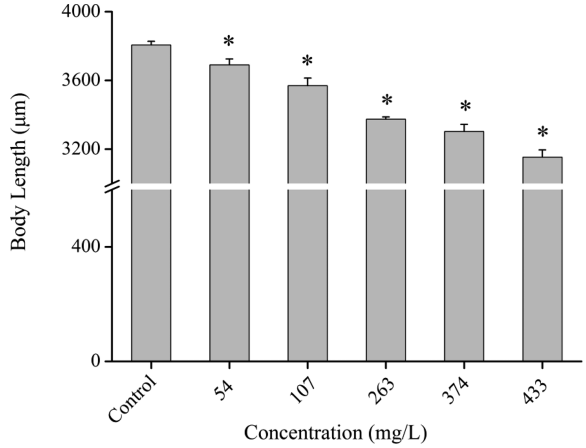

Figure 3. Length of zebrafish larvae (μm, mean ± SEM, $n = 3$) after exposure (120 hpf) to acetamiprid at 0, 54, 107, 263, 374, and 433 mg/L. An asterisk (*) signifies a significant difference ($p < 0.05$) compared with control.

Behavioral Effects. In the behavioral bioassays (clutches 4 and 5, spontaneous movement and tail and head touch responses, respectively), survival was 100% for all embryos (Table S2). Malformed organisms were excluded from behavioral analysis. Spontaneous movements of the embryos followed a sigmoidal response up until 27 hpf wherein the movements started to decrease in both controls and treatments, thus EC50s were recorded only during this time frame (from 17 to 27 hpf; Figure 4). In the controls, no spontaneous movements for the embryos were observed before 21 hpf. The movements gradually increased at 21−23 hpf, reached a

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

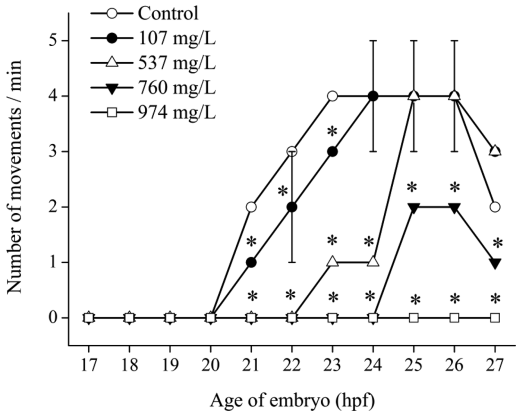

Figure 4. Spontaneous movement analysis of zebrafish embryos (number of movements per min, mean ± SEM, $n = 3$) after exposure (17–27 hpf, hourly) to acetamiprid at concentrations of 0, 107, 537, 760, and 974 mg/L. An asterisk (*) signifies a significant difference ($p < 0.05$) compared to control.

plateau at 23–26 hpf, and then decreased at 27 hpf. Comparatively, in acetamiprid treatments, the emergence of spontaneous movement of the embryos was significantly postponed and even disappeared at the highest concentration. The extent of movement for the embryos exposed to acetamiprid at lower concentrations eventually reached control level with some delays, yet the embryos in higher concentrations (≥760 mg/L) of acetamiprid were not able to recover and did not attain the same response as control organisms at any exposure time. In fact, no spontaneous movement was noted for the embryos exposed to 974 mg/L of acetamiprid (Figure 4). The EC5 and EC50 were calculated for spontaneous movement at various time points (Table S2). At 22–25 hpf, EC50 values for spontaneous movements gradually increased every hour with values of 121 (0–>974), 296 (167–523), 561 (222–1421), and 740 (668–819) mg/L, respectively (Table S2). The effects of acetamiprid exposure on tail and head touch responses were much less sensitive than spontaneous movements (Figures 4 and 5). Comparatively, the impact of touch response to the head was more pronounced than the tail following acetamiprid exposure. Significant

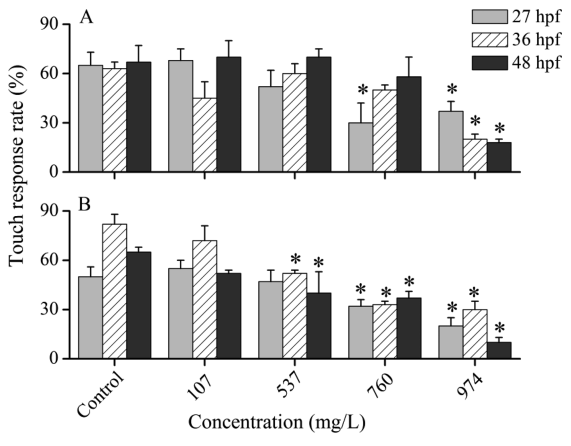

Figure 5. Tail (A) and head (B) touch responses (%, mean ± SEM, $n = 3$) of zebrafish embryos after exposure (27, 36, and 48 hpf) to acetamiprid at concentrations of 0, 107, 537, 760, and 974 mg/L. An asterisk (*) signifies a significant difference ($p < 0.05$) compared to control.

differences for tail and head touch responses between the treatments and the control were only noted for the treatment with the highest concentration (974 mg/L) at all time points, while the response of head touch was observed in the embryos exposed to acetamiprid at 537 and 760 mg/L.

DISCUSSION

Response Spectra and End Point Sensitivity. As shown in Figure 6, a response spectrum was constructed to rank the sensitivity of the tested end points for zebrafish embryos when they were exposed to acetamiprid (the effect concentrations are reported in Table S2). Spontaneous movement was the most sensitive end point tested with an EC5 of 51 mg/L at 23 hpf, followed by reduced heart rate at 72 hpf (EC5: 57 mg/L), impaired growth (body length EC5: 93 mg/L), malformations (EC5: 195 mg/L), and lethality at 120 hpf (LC5: 272 mg/L). Accordingly, the lowest EC50 value was also for spontaneous movement at 23 hpf with a value of 296 mg/L. On the contrary, the highest EC50 was for tail touch response at 48 hpf (888 mg/L).

The influence of xenobiotic pollutants on movement ability in zebrafish is most likely due to a disruption to nervous system.[24] In early developmental stages of the zebrafish, spontaneous movement is regarded as the first motor activity. The spontaneous movement is believed to be a result of uncontrolled action potential by the motoneurons.[24] This development followed the joint development of muscular and motoneuron systems which would also further evoke frequent spontaneous movements in the embryos.[11] As this is one of the first behavioral mechanisms in zebrafish to be developed, it is not surprising that it was the most sensitive end point tested.[25]

As noted, the initiation of spontaneous movement was adversely affected by acetamiprid at all concentrations. Recovery, although delayed, was also evident at concentrations of 107 and 537 mg/L (as motor activity did not differ from the control at later hpfs). This, however, was not the case for larval fish exposed to higher concentrations of acetamiprid at 760 and 974 mg/L, suggesting that acetamiprid halted the development of motoneuron systems in the zebrafish. This observed effect may be associated with the lack of butyrylcholinesterase in zebrafish.[26] Acetylcholinesterase is responsible for the hydrolysis of acetylthiocholine and butyrylthiocholine, and as acetamiprid is a nAChR agonist, it would disturb acetylcholinesterase activity.[2] As such, the lack of butyrylcholinesterase and this mode of action of acetamiprid might account for the profound effect on the motoneuron system in the development of zebrafish embryos.

Exposure to acetamiprid also reduced fish heart rates in all test concentrations. Previous studies on organophosphate insecticides suggested that continuous stimulation of the acetylcholine receptor in zebrafish led to a significant decrease in heart rate of the exposed individuals.[27] Similar results are expected with acetamiprid, as neonicotinoid insecticides act as nAChR agonists.[2] Yamauchi et al.[28] reported that malformations in the pericardium were one of the main causes for heartbeat and blood circulation abnormalities in fish, and this might be a reason for the reduced heart rates observed in the present study as well.

Impaired growth was another sensitive end point tested as significant reduction of body length was noted for all acetamiprid exposure groups when compared with the control. This developmental end point is easily to be measured and is linked to various molecular and cellular responses, thus it has

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J. Agric. Food Chem. XXXX, XXX, XXX–XXX

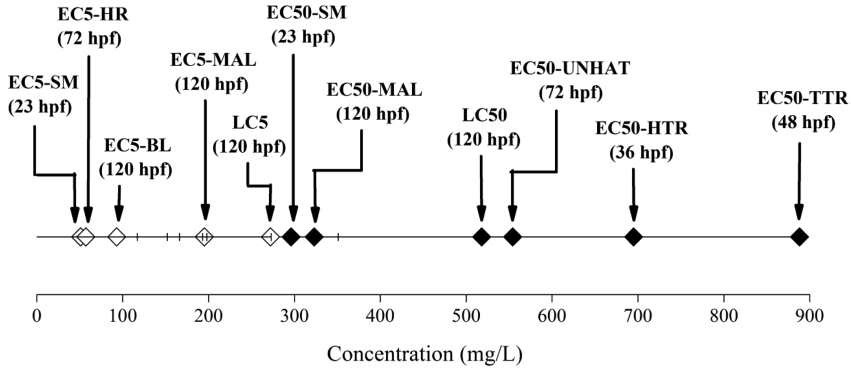

Figure 6. Response spectra of tested end points including sublethal (malformation, lack of hatchability, heart rate, body length, spontaneous movement, and tail and head touch responses) and lethal responses for zebrafish embryos exposed to acetamiprid. Dose metrics are expressed as the concentrations of acetamiprid. EC5 = 5% effective concentration; EC50 = median effective concentration; LC5 = 5% lethal concentration; LC50 = median lethal concentration; BL = body length; SM = spontaneous movement; HR = heart rate; MAL = malformations; UNHAT = unhatched; HTR = head touch response; TTR = Tail touch response. The black squares represent EC50 and LC50 and the open squares represent EC5 and LC5.

been recommended as overall evaluation for the decline of individual fitness.[14] Acetamiprid may directly impact the growth of zebrafish via disrupting synthetic processes of amino acid and/or glucose metabolism.[14] Meanwhile, observed malformations (such as bent spine, uninflated swim bladder, and pericardial edema) could have negatively affected the growth of zebrafish as well.

A variety of morphological abnormalities, such as bent spine, uninflated swim bladder, pericardial edema, and yolk sac edema, were observed in zebrafish embryos exposed to acetamiprid. Although various abnormalities were recorded following exposure to high concentrations of acetamiprid, the most common malformation was bent spine, which occurred in 100% of individuals at 120 hpf at 760 mg/L. Spine malformations have been previously noted for embryos exposed to neurotoxicants, such as fipronil (a neurotoxic insecticide), and have been shown to impair swimming function in larval fish.[29]

Another factor that could affect swimming function is toxicological effects regarding the swim bladder. Goolish and Okutake[30] reported that zebrafish larvae must swim to the water surface to open their swim bladder after breathing air. As discussed above, acetamiprid exposure inhibited spontaneous movements of zebrafish embryos and caused body truncation and other malformations, which reduced swimming ability. As a consequence, larval fish were not able to swim normally to water surface to breathe air, leading to an uninflated swim bladder. The swim bladder is essential for fish development and serves a critical function in ensuring locomotion and buoyancy of larval fish.[31] The noted defects in swim bladders as well as spine malformations would affect the ability of fish to prey on food and escape from predators, most likely leading to death of the organisms.[32]

The TI is a measure of teratogenic potential of a toxicant. If TI for a given substance is higher than 1, the substance is regarded to be teratogenic, which suggests the toxicant has a higher probability of causing serious malformations rather than mortality. On the contrary, when a substance has a TI lower than 1, it may cause death of the fish with little to malformations being observed.[33,34] In the present study, the TI value was 1.6 for acetamiprid, suggesting moderate teratogenic activity of this insecticide to zebrafish embryos.

Hatching rate has been frequently used in assessing embryo development toxicity; however, it was not a sensitive index in the present study as the EC50 for hatching rate was even greater than the LC50. Hatching appeared to be enhanced at a lower concentration (54 mg/L), while being significantly inhibited at higher concentrations (≥537 mg/L). Increasing hatching rates of zebrafish embryos after exposure to acetamiprid at low concentrations might be a result of increased enzyme activity responsible for hatching under stress. The proteolytic hatching enzyme plays a significant role in digesting the chorion during the hatching process for teleost embryos and could have been effected by exposure to acetamiprid.[35] Conversely, hatching rate decreased at higher concentrations of acetamiprid. Several reasons might be linked to the delays in hatching after exposure to acetamiprid, such as adverse effects to neurotransmitters[36] and/or weakening of spontaneous muscular movements.[35]

Zebrafish embryos started to respond to touch as earlier as 21 hpf and continued to response thereafter. At 27 hpf, a touch of the tail would make the dechorionated embryos coil partially, with a brief swimming episode which has been observed in other studies as well.[20] In general, Rohon-Beard sensory neurons in spinal cord are promptly activated to react to tail touch[20] and perception of head stimulation is adjusted by trigeminal neurons.[37] In addition, an intact hindbrain has also been noted as an essential development for the touch response in zebrafish.[18] Therefore, effects to the touch response were evaluated at 27, 36, and 48 hpf. While spontaneous movement was the most sensitive end point, this behavioral effect (both head and tail touch responses) was the least sensitive of all end points tested and only showed adverse effect to zebrafish at the highest acetamiprid concentration tested. It should be noted that although both were rather insensitive, head touch response was more sensitive than the tail touch. This is likely due to the fact that the tail touch would only activate a small number of Mauthner cells, yet head touches activate more reticulospinal neurons which would induce a more significant response.[38] Overall, exposure to acetamiprid showed significant developmental toxicity in zebrafish embryos with various effects on behavior, growth, morphology, hatchability, and death at high concentrations. Evaluating spontaneous movement as an end point was the most sensitive of those tested, but further work is

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

still warranted to better understand the developmental toxicity of neonicotinoids to vertebrates.

Neonicotinoid Toxicity to Fish and Invertebrates. To date, little information is available for toxic levels of most neonicotinoids to fish, regardless of the stage of development (embryo, juvenile or adult). In the present study, the EC50 values for malformations and lethality at 120 hpf of acetamiprid for zebrafish embryos were 323 (303−344) and 518 (469−572) mg/L, respectively (Table S2). While these effect levels are similar to other neonicotinoids in some circumstances, variations among individual neonicotinoids exist. For instance, a previous study on a formulated imidacloprid product found that the 48 h EC50 values for various malformations (missing blood flow, missing body pigmentation, incomplete ear development, missing eye pigmentation, and incomplete eye development) of zebrafish embryos ranged from 408 to 760 mg/L and the 48 h LC50 value was 502 mg/L.[39] These 48 h values for imidacloprid were comparable to the 120 h data for acetamiprid in the present study, suggesting the toxicity of imidacloprid is probably higher than acetamiprid for this fish species. Differences likely exist for embryo toxicity tests between species for the same compound as well, as Tyor and Harkrishan[40] showed an even greater degree of sensitivity with common carp embryos (*Cyprinus carpio* L.) when exposed to imidacloprid, with a reported 48 h LC50 of 78 mg/L. In that same study, researchers showed the viability of fish embryos were significantly dropped at concentrations as low as 7.8 mg/L after 12 h exposure. When exposing to another neonicotinoid, thiacloprid, at a concentration of 0.45 mg/L, behavior, hatching, and embryo viability of common carps showed no difference from the control.[41] The stark differences noted above for embryo toxicity tests between zebrafish and carp for a given neonicotinoid and varying toxicity between individual neonicotinoids for a given species indicated a great need for further research in the area.

While little information is available for embryotoxicity, the toxicity of neonicotinoids, including acetamiprid, to adult fish has been studied. Ge et al.[42] found that imidacloprid caused oxidative stress and DNA damage to zebrafish liver at a concentration of 0.3 mg/L. Similar responses of fish to acetamiprid were also reported by Alam et al.,[43] who demonstrated that for a freshwater fish, *Labeo rohita*, acetamiprid at concentrations of 10−15 mg/L caused a significant decrease in calcium, phosphate, and albumin in blood serum and a significant increase in blood urea. Sublethal concentrations of acetamiprid were also shown to increase the content of amino acids in the head, serum, and liver of adult zebrafish as a consequence of inducing oxidative stress, inhibiting protein synthesis, and inducing DNA and RNA damage. This finally resulted in uridine and adenosine accumulation.[44] In addition, lethality of imidacloprid to adult fish has also been assessed. The reported 96 h LC50s of imidacloprid for rainbow trout, carp, sheepshead minnow, and zebrafish were 211, 280, 161, and 241 mg/L, respectively.[39,40] Lack of available data even in regards to adult fish for acetamiprid emphasizes the need for further studies evaluating the toxicity of this compound to aquatic vertebrates.

Compared with data on adverse effects of acetamiprid on aquatic vertebrates, more information is available for toxicity of this insecticide to aquatic invertebrates. Mo et al.[45] showed that acetamiprid exposure caused delay in larval development and decreased pupa weight of *Culex pipiens pallens*, with 72 h LC50 values being 0.020 and 0.296 mg/L for the first and the fourth instar larvae, respectively. Similar levels of toxicity were reported for *Hexagenia* spp. (mayfly larvae), which had a 96 h LC50 value of 780 μg/L.[46] Comparatively, acetamiprid was less toxic to*Daphnia magna* compared to other invertebrates, and Bownik et al.[47] reported changes in the behavior and physiology of *D. magna* after exposure to Mospilan 20 SP (containing 20% acetamiprid) at relatively high concentrations (25, 50, and 100 mg/L). Regardless of species, aquatic invertebrates are more susceptible to neonicotinoids than vertebrates. It is not surprising as neonicotinoids were developed to bind more strongly to nAChR in the central nervous system in invertebrates than in vertebrates.[3]

Environmental Relevance. Neonicotinoid residues have been frequently detected in surface waters worldwide,[4,9] and acetamiprid is no exception. Reported field concentrations of acetamiprid in water generally ranged from not detected to approximately 100 ng/L in various countries and regions,[48,49] but with a few higher levels being detected more recently, for example, 380 ng/L in rivers near Sydney, Australia[50] and 44100 ng/L in the playa lakes in cropland basins of the Southern High Plains of the United States.[51] It should be noted that the concentrations found in the field, although with high frequency, were a magnitude of difference lower than the concentrations needed to invoke acute developmental effects to zebrafish embryos (and other fish species as discussed above). However, this should not be misconstrued to suggest that these chemicals do not cause adverse effects to aquatic species in the environment. Further work evaluating chronic effects of neonicotinoids to vertebrates (as well as invertebrates[46,52]) is still highly needed to understand the effects of these compounds on nontarget species at environmentally relevant concentrations.

The present study provides a foundation for future work in evaluating chronic toxicity of acetamiprid in vertebrate species, such as fish, which have been commonly overlooked. Outside of aquatic work, the study also provides the basis for extrapolations to other vertebrate species and humans. Studying teratogenic effects, developmental toxicity, and mode of action of chemicals using zebrafish as a model organism have in the past been used to simulate human embryonic development and associated abnormalities and birth defects.[10] Results from studies such as the present one and the extrapolation of acetamiprid to human health become more apparent when one considers that the detection frequency of this compound in fruits and vegetables continues to increase globally.[53]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b05373.

Dose response relationships for mortality, malformations, and hatchability of zebrafish exposed to acetamiprid and measured and nominal water concentrations of acetamiprid in the various bioassays conducted; information regarding the various end points and effect concentrations are also outlined. (PDF)

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

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Notes

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■ ABBREVIATIONS USED

CCD, charge-couple device; EM, embryo medium; hpf, hour post fertilization; nAChR, nicotinic acetylcholine receptor; SEM, standard error of mean; TI, teratogenicity index

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