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ORIGINAL RESEARCH PAPER

# Acute toxicity of neonicotinoids and some insecticides to first instar nymphs of a non-target damselfly, *Ischnura senegalensis* (Odonata: Coenagrionidae), in Japanese paddy fields

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**Abstract**

Systemic insecticides such as neonicotinoids and fipronil are widely applied in rice production. These insecticides have been suspected of reducing biodiversity in paddy ecosystems and reducing wild dragonfly populations in Japan. Conventional ecotoxicological risk assessment could not confirm this, as it has not considered interspecific variation in sensitivity to insecticides. We estimated the median effect concentration (EC50) of 15 systemic insecticides to first instar nymphs of a Japanese damselfly, *Ischnura senegalensis* (Rambur) (Odonata: Coenagrionidae), commonly found in rice paddy fields. Damselflies were found to be highly sensitive to pyrethroid pesticides, less so to phenylpyrazole, organophosphates, and carbamates, and least sensitive to neonicotinoids, nereistoxin, and diamide. Given the acute toxicity data, the sensitivity of the damselfly to neonicotinoids was considered to be lower than that of other aquatic insects, whereas the EC50 values of the damselfly were 2–3 orders lower than that of *Daphnia magna* Straus (Diplostraca: Daphniidae), which is a standard test species. These results indicate that the conventional ecological risk assessment based on acute toxicity data of *D. magna* would underestimate the impact of neonicotinoids on Odonata diversity in paddy ecosystems. We therefore recommend using the paddy-dwelling damselfly as a new test species for insecticide bioassay.

**Keywords** Damselfly · Pesticide · Acute toxicity · Non-target organisms · Biodiversity

## Introduction

Neonicotinoids and fipronil (phenylpyrazole) are classes of insecticide developed in the 1990s (Hainzl and Casida 1996; Sheets 2002). These are systemically acting insecticides that are mainly applied as granules to the soil or as coating agents during crop planting, and their application has expanded worldwide (Simon-Delso et al. 2015). Environmental residues of these insecticides are suspected of negatively impacting non-target organisms in the ecosystem (Sánchez-Bayo 2014). Many in vitro studies have emphasized the lethal and sublethal impacts of neonicotinoids and fipronil on non-target organisms (Pisa et al. 2015).

In Japan and other Asian countries, the use of systemic neonicotinoids and fipronil in rice cultivation has expanded in nursery boxes, water surfaces, and foliage. Insecticides absorbed into the roots of rice prevent damage by sap-sucking, leaf-gnawing, and mining insect pests such as hemipterans, coleopterans, thysanopterans, and lepidopterans during the growth stage. In fact, insecticides used in nursery boxes are the most marketed among Japanese paddy insecticide products (Jinguji et al. 2009). Application to nursery boxes is labor-saving and efficient, and is safer for farm workers (Jinguji et al. 2009). However, the residues of insecticides dissolve in surface water and diffuse into sediment after transplanting the rice to paddy fields. In Japan, neonicotinoids and fipronil applied to paddy fields are suspected of causing population reduction of non-target insects, especially dragonflies (Odonata) (Uéda and Jinguji 2013). Dragonflies are regarded as indicator species to assess the quality of aquatic environments (Kadoya et al. 2011).

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Some field experiments have examined the environmental impact of using neonicotinoids and fipronil as nursery box insecticides on non-target dragonflies in Japanese paddy fields. In a micro paddy lysimeter, imidacloprid treatment reduced the individual numbers of emerged adults of the dragonfly *Sympetrum infuscatum* (Selys) (Odonata: Libellulidae) compared with control, and treatment with fipronil led to no adult emergence (Jinguji et al. 2013). In paddy mesocosm experiments, fipronil had a stronger negative impact on community structure of dragonflies than neonicotinoids did (Hayasaka et al. 2012, 2013a; Kasai et al. 2016). Between two neonicotinoid treatments, environmental impacts on dragonflies differed (Kobashi et al. 2017). Although many insecticides are used in rice paddy fields, the toxic effects of each insecticide on non-target dragonflies are uncertain.

The ecotoxicity of pesticides is qualitatively assessed according to Organization for Economic Cooperation and Development (OECD) (2004) methods on the basis of threshold values calculated from acute toxicity data of three test organisms: fishes, e.g., *Cyprinus carpio* Linnaeus (Cypriniformes: Cyprinidae); daphnids, e.g., *Daphnia magna* Straus (Diplostraca: Daphniidae); and green algae, e.g., *Pseudokirchneriella subcapitata* (Korshikov) Hindák (Sphaeropleales: Selenastraceae). Under this framework, *D. magna* is assumed to be representative of all of primary consumers and aquatic arthropods. However, *D. magna* is considerably less sensitive to neonicotinoids compared with insects (Beketov and Liess 2008; Yokoyama et al. 2009). This difference in sensitivity indicates that the conventional test guidelines are unsuitable for assessing the ecological risk posed by neonicotinoids for many insects (Pisa et al. 2015). Furthermore, *D. magna* is not a native species in Japan, and as such is not representative of typical aquatic organisms of Japanese inland waters and inappropriate for use in local environmental impact assessments. In order to resolve these issues, researchers have developed species sensitivity distribution (SSD), which statically assesses the risk of pesticides using acute toxicity data of multiple species (Nagai 2016). In Japan, a native net-spinning caddisfly, *Cheumatopsyche brevilineata* (Iwata) (Trichoptera: Hydropsychidae), was developed as an insecticide bioassay for non-target insects in lotic environments (Yokoyama et al. 2009), and has been added to the standardized SSD protocol in Japan (Nagai 2016). However, there has not been an insecticide bioassay for non-target native insects in Japanese rice paddy fields.

In this study, we developed a bioassay of a damselfly species, *Ischnura senegalensis* (Rambur) (Odonata: Coenagrionidae), for assessing the ecotoxicity of chemicals on non-target aquatic insects living in the paddy ecosystem. The damselfly is native to Japan and to tropical and subtropical areas of Asia and Africa (Sharma and Clausnitzer 2016). Nymphs of *I. senegalensis* inhabit ponds and slow-stream water bodies, and are commonly found in and around rice paddy fields in Japan. We investigated the acute toxicity of 15 insecticides, including neonicotinoids and fipronil, to damselflies. We then compared the median effect concentration values (EC50) of the damselfly to those of other aquatic organisms.

## Materials and methods

### *Ischnura senegalensis*

We used first instar nymphs of *I. senegalensis* to estimate acute toxicity of insecticides. Adults of a stock strain of *I. senegalensis* initially collected from a river in Ibaraki Prefecture, Japan in 1993, and stored at the National Institute for Environmental Studies over 61 generations, were used to produce nymphs. This line of *I. senegalensis* is maintained by the initially captured population and naive to the pesticides used in this study, precluding the evolution of resistance. Nymphs were fed rotifers, water fleas, and midge larvae. As they grew, they were given larger foods. Adults of *I. senegalensis* were fed adult midges. Food organisms were bred without any long-term pesticide exposure. The nymphs used in the acute toxicity test were prepared as follows: eggs laid on wet filter paper by females were preserved at 18 °C for 3 weeks and then incubated at 23 °C until hatching. First instar nymphs hatched within 24 h were used in the acute toxicity experiment.

### Insecticides

We tested the acute toxicities of 15 insecticides categorized into six groups according to the IRAC Mode of Action Classification Scheme, version 8.2 (IRAC 2017) (Table 1), using analytical-grade chemicals (Wako Pure Chemical Ind., Osaka, Japan). Purity of the insecticides ranged from 98.1% to 100%. On the basis of preliminary experiments, stock liquid preparations were made by dissolving insecticides in acetone, except cartap hydrochloride that was dissolved in ultra pure water. These stock solution had 1000 times the concentration of the test solution (Table 1). These were diluted using deionized aerated tap water (average pH 7.4 ± 0.3 and dissolved oxygen concentration [DO] 7.1 ± 0.8 mg/l) for use in testing.

### Acute toxicity test

All manipulations for acute toxicity testing were conducted at room temperature (20 °C). To avoid photodegradation of insecticides, all handling of insecticides was conducted in a darkened room with indirect illumination by a desk lamp.

**Table 1** Median effective concentration (EC50 in 24 h insecticide exposure and 48 h post-treatment effect) of *Ischnura senegalensis* first instar nymphs to 15 insecticides applied to rice paddy fields in Japan

| Insecticide categorization of IRAC | Class | Insecticide | Purity (%) | Individuals per concentration | Test concentration (μg/l) | Replicates | EC50 (μg/l) | 95% confidence interval |
|---|---|---|---|---|---|---|---|---|
| 1A | Carbamate | Benfuracarb | 98.5 | 5 | 10, 20, 30, 40, 50 | 3 | 28.29 | 24.32–32.26 |
| | | Fenobucarb (BPMC) | 99.7 | 5 | 3.13, 6.25, 12.5, 25, 50 | 3 | 43.61 | 34.19–53.03 |
| 1B | Organophosphate | Fenitrothion (MEP) | 99.2 | 5 | 5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 15 | 6 | 7.871 | 7.396–8.346 |
| 2B | Phenylpyrazole | Fipronil | 98.1 | 5 | 0.5, 1, 2, 4, 8 | 3 | 1.835 | 1.423–2.246 |
| 3A | Pyrethroid | Etofenprox | 99.2 | 5 | 0.05, 0.2, 0.4, 0.6, 0.8, 1 | 3 | 0.6474 | 0.5431–0.7517 |
| | | Silafluofen | 99.7 | 5 | 3.13, 6.25, 12.5, 25, 50 | 3 | 8.193 | 4.585–11.801 |
| 4A | Neonicotinoid | Acetamiprid | 99.9 | 5 | 50, 100, 200, 400, 800 | 3 | 336.5 | 246.2–426.8 |
| | | Clothianidin | 99.6 | 5 | 12.5, 25, 50, 100, 200 | 3 | 121.3 | 91.8–150.8 |
| | | Dinotefuran | 99.9 | 5 | 62.5, 125, 250, 500, 1000 | 3 | 523.3 | 343.5–703.1 |
| | | Imidacloprid | 99.1 | 5 | 1, 25, 50, 75, 100, 125, 150 | 3 | 112.3 | 89.8–134.8 |
| | | Nitenpyram | 100 | 5 | 50, 100, 200, 400, 800 | 3 | 550.6 | 410.0–691.2 |
| | | Thiacloprid | 98.1 | 5 | 25, 50, 100, 200, 400 | 3 | 128.7 | 97.3–160.1 |
| | | Thiamethoxam | 99.8 | 5 | 62.5, 125, 250, 500, 100, 2000, 4000 | 3 | 1372 | 978–1765 |
| 14 | Nereistoxin | Cartap hydrochloride | 99.9 | 5 | 125, 250, 500, 1000, 2000 | 3 | 1053 | 722–1383 |
| 28 | Diamide | Chlorantraniliprole | 98.4 | 5 | 125, 250, 500, 1000, 2000 | 3 | 910 | 576–1243 |

Categorization of insecticides according to IRAC (2017). 1A and 1B, acetylcholinesterase (AChE) inhibitors; 2B, GAGA-gated chloride channel blockers; 3A, sodium channel modulators; 4A, nicotinic acetylcholine receptor (nAChR) competitive modulators; 14, nicotinic acetylcholine receptor (nAChR) channel blockers; 28, ryanodine receptor modulators

Test solutions of five to nine different concentrations were prepared from the diluted stock for each insecticide, with 0.1% (v/v) solvent concentration (Table 1). Ten milliliters of each solution was placed in a 65-ml glass container without bottom material.

One newly hatched (<24 h) *I. senegalensis* swimming nymph was placed in each solution to avoid cannibalism between nymphs in a shared environment. Five nymphs were exposed at each test concentration during 24 h. Tests were replicated three times per insecticide, with the exception of fenitrothion, which was replicated six times (Table 1). Two control groups were prepared, one containing only tap water and one containing tap water with added acetone solvent at an equivalent concentration; each group included 8.9 and 8.5 individuals on average, respectively. Containers were sealed using polyvinylidene chloride film and incubated at 20 °C in a darkened room. Dissolved oxygen concentration and pH of the test solution were measured at the beginning and the end of the test. Nymphs were not given any food during this period.

After 24 h, test solutions were removed from all containers and replaced with fresh deionized tap water. Nymphs were incubated for a further 24 h as above. At this point, nymphs were observed using a stereomicroscope (SZ61, Olympus Co., Tokyo). Their state was categorized as either immobilized (either dead or inactive, even when stimulated with a glass pipette) or alive (swimming or responsive to stimulus).

The estimation of EC50 in each insecticide was carried out using a generalized liner model (GLM) with binomial error and logit link. Likelihood ratio tests were used to test for the effects among replications of fenitrothion. All calculations were conducted in R 3.31 (R Development Core Team 2016). EC50 and standard error were estimated using the dose.p function in the MASS library (Venables and Ripley 2002).

### Comparison of sensitivities in *I. senegalensis* and other aquatic arthropods

To compare the sensitivity of *I. senegalensis* with that of other arthropods, we obtained EC50 and lethal concentration (LC50) data from the observation of aquatic insects and *D. magna* for 2 days, from the ECOTOX database (US Environmental Protection Agency 2017) and from the *Noyaku Handbook*, 13th edition (Japan Plant Protection Association 2011). We downloaded aquatic insects' data for each insecticide from the ECOTOX database. The data were then narrowed to contain only records from aquatic insects obtained in 2 days from laboratory experiments, and including EC50 and LC50. When the same insect species had multiple EC50 or LC50 values in a cited article, only one value was selected; this was the nearest value to the median of the value presented in the article.

## Results

Dissolved oxygen and pH of test solutions were shown to be relatively constant throughout the testing period, suggesting that the observed effects were due to the pesticide treatments. Control nymphs were mostly healthy at 48 h, showing an immobility rate of 0.06 ± 0.09 (mean ± SD) % in the tap water-only control group and 0.03 ± 0.06 (mean ± SD) % in the 0.1% acetone solution control group.

The reproducibility of this insecticide bioassay was verified in six replicated tests of fenitrothion to ensure that there was no effect among replications (ANOVA, $\chi^2 = 7.491$, $p = 0.187$). Therefore, we estimated the EC50 of each insecticide without discrimination among replications.

Acute toxicity of each insecticide to *I. senegalensis* nymphs varied (Table 1), with a 2000-fold difference between the minimum EC50 and the maximum EC50. The lowest toxicity was observed in the neonicotinoid thiamethoxam, followed by the nereistoxin cartap hydrochloride and the diamide chlorantraniliprole. A number of other neonicotinoids, including nitenpyram, dinotefuran, acetamiprid, thiacloprid, clothianidin, and imidacloprid followed. Of considerably greater magnitude were the carbamates benfuracarb and fenobucarb, the pyrethroid silafluofen, the organophosphate fenitrothion, and the phenylpyrazole fipronil. The greatest toxicity was seen in the pyrethroid etofenprox.

In carrying out a literature search, we found more than one toxicity dataset for aquatic insects corresponding to the 15 tested insecticides. The toxicity data of aquatic insects included one Coleoptera, 19 Diptera, four Ephemeroptera, one Hemiptera, one Megaloptera, three Odonata, five Plecoptera, and two Trichoptera species. A scatter plot showed that the sensitivity of *I. senegalensis* to carbamates, organophosphate, phenylpyrazole, and pyrethroids approximates those reported for other aquatic insects (Fig. 1). The sensitivity of *I. senegalensis* to neonicotinoids was lower than that of 88.9% comparable aquatic insects, and its sensitivity to chlorantraniliprole was lower than that of 75% comparators (Fig. 1). Its sensitivity to nereistoxin was considerably lower than that of other aquatic insects. The sensitivity of *I. senegalensis* to neonicotinoids was higher than that of *D. magna* (Fig. 2).

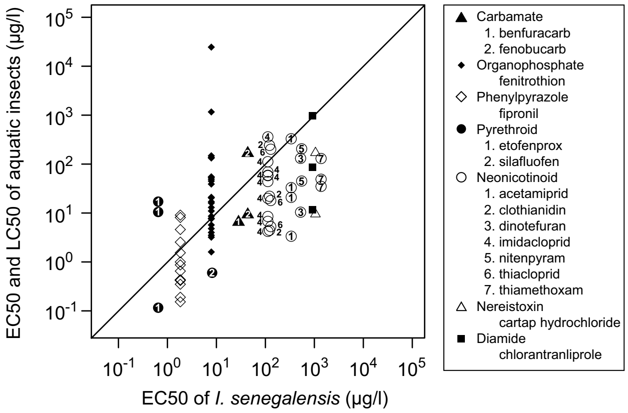

**Fig. 1** Differences in the sensitivities to 15 insecticides (six classes) between nymphs of *Ischnura senegalensis* and other aquatic insects. Figures in or near plot points correspond to insecticide names. Overall, neonicotinoids and diamide insecticides had lower acute toxicity compared than other insecticides

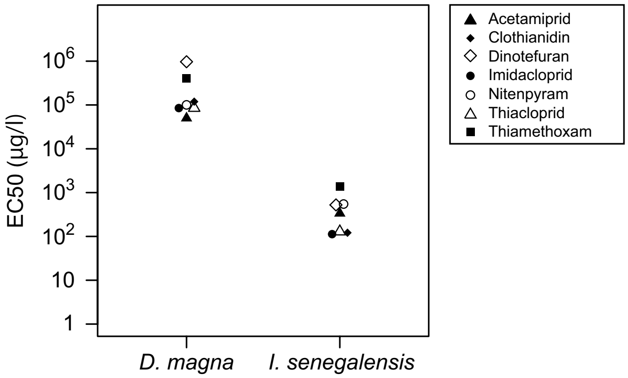

**Fig. 2** Difference in acute toxicity of neonicotinoids between two aquatic arthropods. The sensitivity of *Daphnia magna* to neonicotinoids was 2–3 orders lower than that of *Ischnura senegalensis* (Japan Plant Protection Association 2011)

## Discussion

In this study, we estimated EC50 with 24 h insecticide exposure and 48 h post-treatment effect of 15 insecticides to *I. senegalensis*, simulating the effects of rice nursery box exposure. The EC50 values reported here should approximate median lethal concentrations, owing to the strict criterion of immobilized or dead individuals observed at 48 h. Control nymphs mostly maintained a healthy state during 48 h, with a survival rate of around 95%. This method is a reliable insecticide bioassay for estimating the acute toxicity of insecticides to nymphs of *I. senegalensis*.

Although some EC50 values overlapped among groups of insecticides, the acute toxicity of insecticides to *I. senegalensis* seems to differ according to the mode of action of the insecticides. Neonicotinoids and nereistoxin, which act as a nicotinic acetylcholine receptor (nAChR) competitive modulator/channel blocker, were mildly more toxic to *I. senegalensis* than to other aquatic insects, as was diamide, which acts on $Ca^{2+}$ channel in muscle (Table 1). Acetylcholinesterase inhibitors, organophosphates, and carbamates were more toxic than nAChR agonist/antagonists. Pyrethroids ($Na^{+}$ channel modulators) and phenylpyrazole (GABA-gated $Cl^{-}$ channel blocker) exhibited the highest acute toxicity to *I. senegalensis* in this study.

When comparing the sensitivities of *I. senegalensis* and other aquatic insects, the methods used to obtain them, such as the type of insecticide, the insect's growth stage, and the sampling site, need to be taken into account (Yokoyama et al. 2009). Diagnostic criteria and exposure duration might also differ during the studies. Sensitivity to fenitrothion and fenobucarb was nearly equal among nymphs of two Japanese Odonata species and other aquatic insects (Katayama et al. 2015). By contrast, a comparison of acute toxicity among freshwater arthropods showed that the sensitivity of the common darter *Sympetrum striolatum* Bartenef (Odonata: Libellulidae) to thiacloprid was the lowest among aquatic insects (Beketov and Liess 2008). Although our results indicate that neonicotinoids are the least acutely toxic to *I. senegalensis* compared to other insecticides (Fig. 1), the acute toxicity of neonicotinoids to *I. senegalensis* is still 2–3 order higher than that observed for *D. magna* (Fig. 2).

The EC50 values for each neonicotinoid were considerably higher than predicted environmental concentrations (PEC) in paddy water (Ministry of the Environment, Japan). However, neonicotinoid concentrations in paddy water rose temporarily owing to nursery box application after rice planting. In two paddy mesocosm experiments, the neonicotinoid concentrations at rice planting after 2 h were 30–49 μg/l for imidacloprid and 2.62 μg/l for clothianidin, with these concentrations decreasing rapidly over the next several days (Hayasaka et al. 2013b; Kasai et al. 2016). In lysimeter experiments, imidacloprid concentration in surface water was 52.8 μg/l at 6 h after rice planting (Jinguji et al. 2013). These concentrations were around 25–50% of the imidacloprid EC50 and 2% of the clothianidin EC50. Although the mesocosm experiments revealed structural changes of dragonfly nymph communities, *I. senegalensis* increased in abundance despite dragonflies decreasing (Hayasaka et al. 2013b; Kasai et al. 2016). These reports do not contradict the low sensitivity of *I. senegalensis* to neonicotinoids found in this study. However, attention should be given to the indirect effect on damselfly nymphs of fluctuating asymmetry, seen after imidacloprid exposure at low concentration (Chang et al. 2007). The sublethal effects of neonicotinoids on *I. senegalensis*, such a decrease in survival rate through diminished locomotion capacity, are also not well characterized. It is necessary to investigate the sublethal effects of *I. senegalensis* for assessing ecotoxicity impacts of neonicotinoids on paddy ecosystems.

The acute toxicity of fipronil to *I. senegalensis* was relatively high among the insecticides tested in this study (Table 1). The EC50 of fipronil (1.835 μg/l) was ca. 53 times higher than its PEC (0.034 μg/l; Ministry of the Environment, Japan), and higher than surface water concentration immediately after rice planting in two paddy mesocosm experiments (< 1 μg/l) (Hayasaka et al. 2012; Kasai et al. 2016). The paddy water concentration rapidly decreased for several days after rice planting. On the other hand, the soil concentration of fipronil was very high several days after rice planting (ca. 10–192 μg/kg), and fipronil is well retained in soil (Hayasaka et al. 2012; Kasai et al. 2016). In the mesocosm experiments, fipronil treatment greatly decreased the number of exuviae of dragonfly nymphs in a paddy compared with control and neonicotinoid treatments. By contrast, the exuviae of *I. senegalensis* increased in fipronil and neonicotinoid treatments regardless of the high acute toxicity of fipronil (Hayasaka et al. 2013a; Kasai et al. 2016). On the basis of the *I. senegalensis* life cycle, the increase in exuviae could be interpreted as resulting from multiple reproductive generations and associated time lags in the migration of females to paddy fields during the summer.

No insecticides were added to the experimental field paddies after rice planting in previous mesocosm studies (Hayasaka et al. 2013a; Kasai et al. 2016), although some insecticides, such as that applied to water surface and foliage, are usually provided to practical paddies in summer. The insecticides used in these applications include the compounds tested in the present study. Although the behavior of each insecticide after its applications is largely unknown, concentrations of water-soluble insecticides in paddy water are expected to increase sharply and then decrease. Pulsatile concentration rise of highly toxic insecticides, such as pyrethroid and organophosphate, can cause serious acute toxicity to summer-hatched *I. senegalensis* nymphs. Thus, the annual insecticide application plan in rice paddies needs to be considered for accessing the effect of insecticides on the wild population of *I. senegalensis*.

In this study, we developed a new reliable insecticide bioassay for estimation of the EC50 of *I. senegalensis*, which lives commonly in paddy water as a non-target aquatic insect. Our results indicate that conventional acute toxicity testing could cause underestimation of the impact of neonicotinoids on aquatic insects in paddy ecosystems, owing to higher sensitivity in damselflies than in daphnids. We suggest that the EC50 value should be applied in SSD, which is important in environmental impact assessment of regions such as Japan, where paddy fields cover a large area. However, we did not examine the sublethal effects of insecticide on *I. senegalensis*. It is necessary to verify the effects of low-concentration pesticides on locomotive ability and survival rates over longer durations in laboratory and field works.

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