Effect of diazinon on life stages and resting egg hatchability of rotifer *Brachionus plicatilis*

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This paper has not been submitted elsewhere in identical or similar form, not will it be during the first three months after its submission to Hydrobiologia.

Abstract

The effects of organophosphate pesticide, diazinon, on life history parameters and hatchability of resting eggs of rotifer *Brachionus plicalitis* were assessed. Newly hatched (<1h-old) neonates were individually cultured in six varying concentrations (0/control, 0.1, 1.0, 2.5, 5.0 and 10.0 mg/L) of diazinon. The life history parameters such as time (h) the rotifers bear first egg and release first neonate, reproductive period, net reproductive rate, mixis, intrinsic rate of population increase, and life span were evaluated. Results showed that among the life history parameters, the time the rotifers took to release neonates is the most sensitive, giving the lowest EC_{50} value of 1.24 mg/L. The fecundity of maternal females, amictic and mictic daughters was also investigated. Rotifers exposed to 10.0 mg/L produced significantly fewer amictic daughters, and at this concentration, rotifers did not produce any mictic daughter. At 5.0 mg/L, the number of male offspring was significantly lower than the control. Furthermore, the hatchability of resting eggs produced by the rotifers was evaluated when exposed to diazinon: from birth until they produced resting eggs (early development); during late developmental stage of resting eggs (before diapause); and during diapausing stage. The hatchability of the resting eggs was not affected when exposure was timed at late developmental and diapausing stages. Overall results showed that even though amictic females reproduced normally in the presence of low concentration of diazinon, sexual reproduction is severely affected, especially the hatchability of resting eggs when the exposure was timed on its early developmental stages. This provides another evidence that production of resting eggs is particularly sensitive to the presence of xenobiotics in the environment.

Introduction

Pesticides and their residues are reported to be among the most devastating agents in the aquatic ecosystem (reviewed by Hanazato, 2001). Significant amounts of pesticides belonging to class organophosphate been formulated and widely used in agricultural areas, households, and urban setting. One of the most widely used organophosphate pesticides is diazinon. Diazinon is known to inhibit acetylcholinesterase, an enzyme responsible for inactivating the neurotransmitter acetylcholine in target and non-target organisms (Ecobichon & Joy, 1994; Pesando et al., 2003). Due to its chemical properties, widespread use and application, diazinon is frequently found in point sources (wastewater treatment plant effluent) and non-point sources (storm water runoff) in urban and agricultural areas, and known to be lethal to many aquatic species (EPA, 2003).

Acetylcholinesterase receptors have been reported in monogonont rotifers (Nogrady & Alai, 1983; Pineda-Rosas et al., 2005). It is more likely, that diazinon can inflict several effects in this important group of zooplankton. Our previous study found that the resting egg hatchability of monogonont rotifer, *Brachionus plicatilis*, is severely affected by the presence of diazinon in their culture medium (Marcial et al., 2005). However, since we employed batch culture method, the individual life history of each rotifer is not clarified.

B. plicatilis incorporates both asexual (amictic) and sexual (mictic) reproduction into their life cycle. Amictic females produce egg, mitotically that develop into amictic females. Under optimum environmental condition for asexual reproduction, amictic females can produce mictic females, the process known as mixis (Snell, 1986; Hagiwara et al, 1988). Mictic females subsequently produced eggs meiotically, which developed

into haploid males, or resting eggs if fertilized by males. Oviposited resting eggs remain attached to the mother 2-3 days before being released. Hagiwara et al. (1995) found that oviposited resting eggs of *B. plicatilis* still undergo some development, thus, it is not considered as true diapausing stage. Starting from day four after oviposition, the number of nuclei and shell layers of resting eggs incubated at 25^{o}C begin to arrest their development, so the authors hypothesized that the resting eggs entered the external diapausing stage. These complex life stages and resting egg developmental stages of *B. plicatilis* can be a good model to investigate the effect of a toxicant in various life cycle stages, as any of these stages may be impaired during toxicant exposure. Furthermore, since resting egg is considered as "seed" for the rotifer to continue their population after harsh environmental condition, the effect of environmental pollutants on its hatchability should be fully assessed.

This study aimed to determine the effect of an organophosphate pesticide, diazinon, on different life stages of individually cultured rotifer *B. plicatilis*, and hatchability of resting eggs when exposure was timed during different developmental stages.

Materials and methods

Test animals

B. plicatilis NH1L strain was used in this study. This strain originated from eel culture pond in Mie Prefecture, Japan, and has been cultured in Nagasaki University, Japan, for 18 years. Stock cultures were maintained in diluted seawater at 22 ppt, stored in 25 ± 1^{o}C, and fed *Nannochloropsis oculata*, with intermittent collection and hatching

of resting egg to start a new culture. Among the *B. plicatilis* stock cultures maintained in our laboratory, this strain has the highest mixis rate (20-60%). Fertilized mictic female of this strain usually spawn or oviposited resting egg 30-33 hours after impregnation, and the oviposited eggs remain attached to the mother 2-3 days before they were release. The three different stages of resting egg development used in the present study were based on the descriptions of Hagiwara et al. (1995): 1) early developmental stage starts from the birth of the parents until the mother produced resting egg; 2) late developmental stage starts from oviposition of the resting eggs until day 4; and 3) external diapause which starts from day 5 after oviposition.

Test chemical and concentrations

Diazinon (WAKO Pure Chemical Industries Ltd., Japan), which is not readily soluble in water, was first dissolved in 100% dimethyl sulfoxide (DMSO), then in Milli Q water (Millipore). The final test solution contained not more than 0.01% DMSO. Stock solution was stored at 4^{o}C until used and renewed every week. Concentrations tested were: 0 (control), 0.1, 1.0, 2.5, 5.0 and 10.0 mg/L. Since 0.01% DMSO did not affect any reproductive parameters of *B. plicatilis* (Marcial et al., 2005), it was not tested in the present study.

Experiment 1. Effect on life history parameters of maternal female

Test animals were obtained by hatching resting eggs. Twelve newly hatched (<1h) amictic females were individually allocated in 24-well polystyrene plates containing 1 mL 22 ppt sterilized diluted seawater with 7×10^6 cells/mL *N. oculata* and one of six concentrations of diazinon. After the addition of rotifers, the plates were kept

at 25°C in darkness. After 20 hours, the plates were inspected hourly to monitor the number of hours the rotifers bear their first egg and release the first neonate (generation time). Thereafter, the plates were inspected once a day, where the maternal female was transferred in another well containing fresh food and toxicant solution. During the transfer, the offspring was counted while female types were classified on the next day based on the description of Hagiwara et al. (1988). From the raw data, generation time, fecundity, reproductive period, and life span were determined, and mixis was calculated. Intrinsic rate of natural increase was calculated according to Lotka equation: $\sum l_x m_x e^{-rx} = 1$, where l_x is the probability of surviving animal at age x, and m_x, the average number of neonates produced per animal at age x.

From each treatment, 12 one-egg bearing amictic female offspring were randomly selected and transferred to another set of plates with food and toxicant solution and used in Experiment 2. In addition, all mictic offspring produced (up to F_3) in each treatment, were also transferred to another set of plates and used in Experiment 2.

Experiment 2. Effect on fecundity of amictic and unfertilized mictic offspring

The amictic and mictic females selected from Experiment 1 were individually cultured in 24-well polystyrene plates containing food and toxicant solution. The plates were inspected daily, and the number of offspring (females and males for amictic and mictic female, respectively) they produced daily was counted and removed. Lifetime fecundity was determined.

Experiment 3. Effect on hatching of resting eggs after exposure to diazinon at different development stage:

a) from birth of the parents to early developmental stage

Test animals were obtained by shaking egg-bearing amictic females in a screw-capped bottle, and hatching the eggs in a 15-mL petri dish containing 10 mL of *N. oculata* suspension. In each treatment, 10 amictic females bearing first laid egg were inoculated in a 20-mL screw-capped bottle containing 10 mL of 7×10^6 cells/mL *N. oculata* and toxicant solution and incubated at 25°C in darkness. Each treatment consisted of three replicate bottles. On days 2 and 4, five mL of the same concentration of food and toxicant solutions were added in each bottle. On days 6, 7, and 8, newly fertilized mictic females were collected and washed twice with sterilized seawater, and transferred to a new bottle containing sterilized diluted seawater (22 ppt) without food and toxicant. Hence, mixis induction, male production, mating, fertilization and earlier embryonic development of resting eggs occurred under diazinon exposure. The bottles were kept at 25°C in darkness for three weeks to permit resting egg development. Under this condition, fertilized females did not produce resting eggs. After incubation, hatching was induced by placing the eggs in a glass petri dish with 10 mL of diluted sterilized seawater (22 ppt) and exposed to continuous light (4000-5000 lux) at 25°C. After 24 hours, the hatched and unhatched resting eggs were counted. The observation of possible hatching was continued until day 4.

***b**) late developmental stage*

Newly hatched females were randomly selected from the stock culture and transferred to 6-well plates containing food suspension. The plates were monitored hourly. As soon as fertilized mictic females were found, they were pipetted out and inoculated into fresh food and toxicant solution. Each treatment received at least 20 females. On day 3, resting eggs at the bottom of the bottle were collected and washed

twice with sterilized seawater and transferred in a bottle containing 10 mL of diluted sterilized seawater (22 ppt). The bottles were kept at 25^{o}C in darkness for three weeks. The same procedure of hatching was employed as described above. The experiment was repeated three times as replicates.

c) diapause stage

Fertilized mictic females (30/treatment) were collected from the stock culture and transferred in 6-well plates containing 7 mL food solution. The plates were inspected hourly. As soon as the females released resting eggs, the eggs were pipetted out and exposed to the toxicant for 7 days at 25^{o}C. On day 8, all resting eggs were washed twice with sterilized seawater, and transferred in a bottle containing 10 mL of diluted sterilized seawater (22 ppt). The bottles were kept at 25^{o}C for three weeks. The same procedure of hatching described above was employed. The experiment was repeated three times as replicates.

Statistical Analysis

One-way analysis of variance (ANOVA), with concentration as the independent variable and number of hours to bear the first egg, number of hours to release the first neonate, fecundity, reproductive period, life span, and mixis as dependent variables, followed by Dunnett's test for pair-wise comparisons of each pesticide concentration relative to the control (Zar, 1999) were conducted. In addition, the concentration of the toxicant that reduces the test parameters to 50% (EC_{50}) was calculated using regression analysis (Stephan & Rogers, 1985). Regression lines were calculated from mean number of hours to bear the first egg, number of hours to release the first neonate, average

number of offspring produced by each female, reproductive period, mixis, life span, intrinsic rate of natural increase and percentage of hatch resting eggs per treatment were plotted against log toxicant concentration. The percent hatching at different pesticide concentration was compared to the control using chi-square contingency test. A *p* value of 0.05 or less was regarded as being significant for all tests.

Results

Effect on life history parameters of maternal female

The life history parameters of control and diazinon-treated rotifers are shown in Table 1. Rotifers exposed to 5.0 mg/L and higher doses of diazinon took significantly longer time to produce eggs, and those exposed to 2.5 mg/L and higher, released their first neonates later than the control. The overall fecundity and reproductive period were significantly lower only at highest concentration tested (10 mg/L), while mictic female production was significantly reduced starting from 2.5 mg/L. No mictic female was observed at 10 mg/L. Life span was significantly affected only at 10 mg/L. Among the life history parameters, the time (h) to release first neonate gave the lowest EC_{50} value (Table 2).

Effect on the fecundity of amictic and unfertilized mictic females

The fecundity of amictic and unfertilized mictic offspring obtained in Experiment 2 is presented in Table 3. Amictic females exposed to 10.0 mg/L produced significantly fewer offspring compared to control and lower concentrations. At 5.0 mg/L, mictic females produced significantly fewer males than the control. The EC_{50} of mictic female

reproduction was lower than that of amictic female reproduction (1.26 mg/L and 1.44 mg/L, respectively; Table 2).

Effect on hatching of resting eggs

Rotifers exposed to two highest concentrations (5.0 and 10.0 mg/L) of diazinon starting from birth did not produce any resting eggs, and at 1.0 and 2.5 mg/L, the hatchability of the resting eggs was significantly reduced (Table 4), however the hatchability was not affected when the rotifers are exposed to diazinon during late developmental and diapausing stages of resting eggs. Hatchability of resting eggs exposed to diazinon during the late developmental stage ranged from 66.8 to 73.5%, while during diapausing stage, from 63.4 to 75.6%. The hatchability of resting eggs exposed from birth to early developmental stage gave the lowest EC_{50} value (0.60 mg/L; Table 2).

Discussion

This study aimed to determine the effects of an organophosphate pesticide, diazinon on life cycle stages of rotifer, *B. plicatilis*. It further aimed to determine the hatchability of the rotifer resting eggs when exposure was timed during different stages of development. Overall results showed that sexual reproduction is more sensitive than asexual reproduction, and the resting egg hatchability is severely affected when rotifers are exposed to the toxicant during its production and/or early developmental stage. Several studies using freshwater rotifer *Brachionus calyciflorus* also found that sexual reproduction is more sensitive parameter than asexual reproduction in the presence of

endocrine disrupters and heavy metals (Preston et al., 2000; Preston & Snell, 2001). Diazinon is also known to affect filtration and ingestion rate of *B. calyciflorus*, giving an EC_{50} of 14.39 and 14.22 mg/L, respectively (Fernandez-Casalderry et al., 1992). These concentrations are two magnitude lower than the EC_{50}s we obtained on different life history parameters of *B. plicatilis* (Table 2).

All of the life history parameters of maternal female were affected at the highest concentration of diazinon (10 mg/L) tested (Table 1). While the fecundity (total number of neonates produced in a lifetime) was significantly lower than the control only at 10mg/L, mictic female production (mixis) was significantly lower starting from 2.5 mg/L. Furthermore, no mixis occurred at 10mg/L. This result is in contrast with our results using batch culture method, wherein mixis occurred even at 10mg/L of diazinon (Marcial et al., 2005). High density and rotifer-conditioned water are among the stimuli that enhanced sexual reproduction in *B. plicatilis* (Snell & Boyer, 1988; Carmona et al., 1993; Hagiwara et al., 1994). These factors are absent in this experiment, since we employed individual culture method. However, using this method, we were able to eliminate possible group interaction, thus the effect could be wholly attributed to the toxicant.

Among the parameters investigated, resting egg hatchability is the most sensitive, resulting to an EC_{50} of 0.60 mg/L (Table 2). Since this parameter is usually neglected in toxicity studies, its inclusion is highly recommended. Among asexual reproduction parameters, generation time or the time to release first neonate is the most sensitive resulting to an EC_{50} of 1.24 mg/L (Table 2). Embryogenesis, the first stage of rotifer life cycle, is also known to be the most sensitive stage to toxicants in other zooplankters including daphnids (LeBlanc, et al., 2000) and copepods (Marcial et al., 2003).

Generation time is a good parameter to investigate toxicant effect on rotifer species that do not produce resting egg.

The fecundity of the amictic and unfertilized mictic female offspring of the diazinon-exposed parent were also compared. Mictic females produced significantly less offspring starting from 2.5 mg/L, while amictic females production was affected from 5.0 mg/L (Table 3). This result supports our conclusion in Experiment 1, that sexual reproduction is more sensitive than asexual reproduction. This result has important implication to resting egg production. Resting eggs are produced when a mictic female is fertilized by males. It is known that *B. plicatilis* does not diffuse mating signals but solely depend on male-female contact in order to mate (Snell & Garman, 1986). A decrease in the number of male, therefore could reduce the chance of male-female contact, thereby reducing resting egg production.

Our previous study showed that the hatchability of resting eggs was severely affected when rotifers were continuously cultured in the presence of pesticides (Marcial et al., 2005). In the present study, we hypothesized that the degree of sensitivity of different stages of resting egg development differs, so we exposed the rotifers during different developmental stages. We found that the hatchability of the resting eggs was affected only when the rotifers are exposed to diazinon during their production and/or early developmental stages. Once they are fully developed and/or already in the diapausing stage, their hatchability is not affected (Table 4). This result is in contrast to the report of Raikow et al. (2006), which found that exposure of *B. plicatilis* resting eggs to biocide SeaKleen for 24 hours resulted to its very low hatchability (24-h LC_{50} = 1.1 mg/L). SeaKleen is a biocide, and it is specifically formulated to kill invasive vector

species including zooplankters' resting eggs present in ballast tanks (Cutler et al., 2004). Therefore, the toxicity of both toxicants differs in magnitude. However, our result clearly showed that the timing of exposure of resting eggs to a toxicant is critical to its viability. The effect of other group of toxicants (e.g. heavy metals, endocrine disrupters) in the hatchability of rotifer resting eggs is worthwhile examining because of their ecological importance in maintaining rotifer population after harsh environmental conditions as discussed in our previous paper (Marcial et al., 2005).

The overall result of this study implies that mictic female production and subsequently resting egg production proceeds only in the presence or low concentration of toxicant in their system. This supports the previous studies which found that mixis in B. plicatilis does not occur in the presence of environmental stress such as high ammonia (Snell & Boyer, 1988), low food (Snell, 1986), and extreme temperature and salinity (Lubzens et al., 1985; Hagiwara et al., 1988). Furthermore, we found that resting egg hatchability is the most sensitive parameter in detecting pesticide effect in rotifer. This endpoint should be included in investigating the effect of any toxicant in this species.

(The overall result of this study reveals that sexual reproduction is more sensitive parameter than asexual reproduction. In addition, mictic female reproduction and subsequently resting egg production proceeds only in the absence or low concentration of toxicant in their system. Concurrent to our previous results (Marcial et al., 2005), we found that resting egg hatchability is the most sensitive parameter in detecting pesticide effect in rotifer. This endpoint should be included in investigating the effect of any toxicant in this species.)

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Table 2. Fifty percent (50%) effective concentration (EC_{50}) values of life history parameters and hatchability of resting eggs of rotifer *B. plicatilis* exposed to diazinon.

Parameter	EC_{50} (mg/L)
Time (h) to bear first egg	1.28
Time (h) to release first neonate	1.24
Fecundity	1.28
Reproductive period	1.52
Life span	1.62
Amictic female reproduction	1.44
Mictic female reproduction	1.26
Mixis	1.26
Intrinsic rate of natural increase	1.53
Resting egg hatchability	
a) early developmental stage	0.60
b) late developmental stage	2.51
c) Diapausing stage	-

- EC_{50} could not be calculated because no correlation was obtained

Table 3. Fecundity of amictic and unfertilized mictic offspring exposed to different concentrations of diazinon. Data are mean and standard deviation. Number in parenthesis is total N.

Concentration (mg/L)	Amictic female	Unfertilized Mictic female
0	15.8 ± 2.2 (12)	15.0 ± 3.5 (12)
0.1	13.7 ± 2.3 (12)	11.3 ± 4.4 (12)
1.0	14.8 ± 2.2 (12)	11.4 ± 3.1 (10)
2.5	15.2 ± 2.8 (12)	13.9 ± 3.2 (5)
5.0	14.8 ± 1.8 (12)	6.7 ± 1.7 (3)*
10.0	6.0 ± 4.8 (12)*	-

- no mictic female was produced

*Significantly different from the control ($P<0.05$) detected by ANOVA and Dunnett's test

Table 4. Hatching (%) of resting eggs when exposure to diazinon was timed during early, late, and diapausing stage. Data are mean and standard deviation (±) of 3 replicates.

Concentration (mg/L)	Early development	Late development	Diapause stage
0	59.1 ± 12.9	72.2 ± 7.9	69.4 ± 3.9
0.1	36.9 ± 10.6	73.5 ± 6.1	74.4 ± 4.2
1.0	11.9 ± 9.3*	69.4 ± 3.9	63.4 ± 10.1
2.5	7.1 ± 4.0*	70.1 ± 1.9	71.2 ± 3.8
5.0	-	68.9 ± 2.6	75.6 ± 12.7
10.0	-	66.8 ± 4.5	68.3 ± 21.2

*Significantly lower than the control ($P<0.05$) detected by chi-square test.
- no fertilized resting eggs were spawned.