

GEORGIA INSTITUTE OF TECHNOLOGY

Rotifer Ecotoxicology: Behavioral Avoidance of Toxicants

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ABSTRACT

Previous rotifer ecotoxicology studies have shown varied effects of sublethal concentrations of hormones and metals on species but have largely ignored toxicant effects on behavior. Given the importance of chemical cues for mating, grazing, and predator avoidance, the phenomenon of behavioral response to pollutants is a critical topic impacting rotifer survival and reproduction. Dual- and tri-chamber test slides similar to Y-tubes were developed to test rotifer behavioral responses to sublethal concentrations of several toxicants. Rotifers were placed in a start chamber between a control chamber and test chamber containing a toxicant, and after fifteen minutes, rotifer distribution in all chambers was recorded. No significant distributional effects were observed for cadmium (2µg/L), pentochlorophenol (2µg/L), flutamide (8µg/L) nor progesterone (8µg/L). Significant deviation from a random distribution was recorded for selenium (2µg/L), lead (8µg/L), and rotifer conditioned medium. In addition, significant avoidance was found for copper (2µg/L) and mercury (0.2µg/L), even in tests with the start chamber containing the toxicant. These data suggest that rotifers can detect and avoid certain toxicants at sublethal levels. Avoidance often occurs at levels below published lethal concentrations (LC50s) on which many water quality criteria are based. Avoidance can alter rotifer survival and reproduction, leading to reductions in rotifer abundance and energy transfer to higher trophic levels.

INTRODUCTION

Rotifers, known since the days of Leeuwenhoek in the late 17th century, are a moderate-sized animal phylum of some 1,850 species of tiny, bilaterally-symmetrical protostomes (Wallace, 2002). Although individually their biomass is minute, because of

their large population size (Wallace, 2002), coupled with high turnover rates due to sexual and asexual reproduction (Snell & Janssen, 1996), rotifers have long been a critical component of aquatic ecosystems despite their limited sensory capability (Wallace, 2002).

Given the age of Rotifera and given that receptors for chemical stimuli are known to be the oldest and most common sensory systems (Dusenbery, 1992), it has been suggested that chemical signals are likely one of the main modes of sensory perception and life cycle regulation in rotifers (Snell, 1998). One such chemically regulated response is the induction of sexual reproduction, or mixis, which occurs in many monogonont rotifer species, including *Brachionus plicatilis* (Stelzer & Snell, 2003). Like other monogonont species, the *B. plicatilis* heterogonic life cycle begins when an amictic female hatches from a resting egg (Gilbert, 2003), which then matures and produces more amictic females via parthenogenesis. As a population increases in density, a chemical cue (the mixis induction protein, Snell et al., 2006) accumulates until it reaches a threshold concentration (Carmona et al., 1993) and induces mixis, as first suggested by Hino and Hirano in 1976 and confirmed by Stelzer and Snell in 2003. Once in mixis, females begin to produce eggs that give rise to mictic daughters who produce mictic eggs, which if unfertilized, develop into haploid males (Hoff and Snell, 1987). These males can then fertilize other mictic females and produce resting eggs (Birky and Gilbert, 1971). These dormant eggs not only serve as a source of genotypic variation and an escape from unfavorable environmental conditions, but also slow the population growth as well (King & Snell, 1977; Snell 1987). Once conditions in the environment are favorable again, the

resting eggs will hatch (Snell, 1987), and the population begins another cycle of increase, sex, and resting egg production.

Because rotifer swimming activity and feeding, along with predator defense, mating, and migration (Wallace, 2002) are characteristics also believed to be triggered by chemical stimuli, the effects of a stimulant on any of these characteristics is of interest. Rotifers have previously been shown to congregate at high population densities within thin layers of algae after sensing the algal food (Ignoffo et al., 2005). Rotifers have also been shown to display varying photo-tactic responses to monochromatic light (Cornillac & Wurdak, et al. 1983). However, additional knowledge of how chemicals transmit information about location, food quality, conspecifics, competitors, and predators is critical for understanding how aquatic ecosystems function and understanding how a potential toxicant can interfere with and affect the ecosystem as a whole (Snell, 1998). Low, non-toxic concentrations of chemicals, ranging from heavy metals and pesticides to seemingly harmless substances, have already been shown to disrupt the transfer of chemical information; this induces maladaptive responses in both the signaler and the receiver (Lürling & Marten, 2007). These behavioral observations not only provide clues about how an organism receives cues, whether by chemicals released into the water or chemicals present on the surface of their food or on other animals (Snell, 1998), it can also reveal factors which limit species distribution, organize aquatic communities, and determine natural selection in aquatic environments (Snell, 1998).

Considering the ease and speed of making quantitative measurements of mortality and reproduction in rotifers (Snell & Janssen, 1995), along with known rotifer sensitivity

to pollutants and their potential ecosystem impact (Wallace, 2002), it follows that rotifers have been used in pollution monitoring and ecotoxicological testing (ET) for some time (Wallace, 2002). In ET, rotifers are exposed to compounds according to standardized protocols, with results reported as LC50s, EC50s, or NOECs for reproductive and/or behavioral endpoints (Wallace, 2002). In this latter method, there is distinct advantage for assessing aquatic toxicity because of how rapidly behavioral responses occur (Wallace, 2002). In addition, the concentrations required to disrupt chemical information systems are typically lower than the concentrations needed to cause other adverse effects, such as mortality (Lürling & Scheffer, 2007).

Two types of rotifer behavioral responses have commonly been chosen to detect toxicity: swimming activity (i.e., speed and sinuosity of swimming) (Charoy & Janssen, 1999) and feeding (Ferrando & Janssen, et al., 1993). In studies using these characteristics, rotifers in the sexual (mictic) stages were shown to be most sensitive to toxicants (Preston & Snell, 2001); however, between species, a comparable sensitivity to most compounds has been shown with no single species consistently the most sensitive to all compounds (Snell & Janssen, 1995). Janssen and Ferrando et al. (1994) have shown clear dose response effects to specific toxicants (copper, pentachlorophenol [PCP], 3,4-dichloroaniline (DCA), and lindane) in the freshwater rotifer *B. calyciflorus*, with rotifer swimming activity decreasing with respect to increasing toxicant concentrations. Ramirez-Perez and Sarma, et al. (2004) have also demonstrated similar effects in swimming activity with mercury. Questions still to be answered include whether or not rotifers can detect toxicants at sublethal levels, whether or not they can avoid toxicants,

and what consequences there are for the rotifer population and the ecosystem at large if such detection and avoidance occurs

Several studies have shown specific rotifer population effects as a result of toxicity. Sarma and Martinez-Jeronimo et al. (2006) discovered that increased ambient metal concentration of cadmium or chromium results in decreased rotifer offspring production. Perhaps more interestingly, Moreno-Garrido and Lubian et al. (1999) have shown that even ingested algal biomass with preaccumulated metal (that is, indirect exposure of rotifers to metal toxicants) caused a delay of 1 or 2 days in rotifer population development. This effect is also seen for both copper and cadmium metals in rotifer species that prey on other rotifers (Gama-Flores & Ferrara-Guerrero, et al. 2007). Cadmium, combined with a naturally competing rotifer species, results in decreased population growth for both competing populations (Gama-Flores & Sarma, et al. 2006). Even an abundance of naturally occurring substances like juvenile hormone and serotonin have been shown to influence populations by increasing mictic (sexual) female production in *B. plicatilis* (Gallardo & Hagiwara, et al. 2000). Still, despite obvious consequences to the population, the question of possible behavioral avoidance of toxicants still goes unanswered.

Perhaps the need for behavioral avoidance studies in rotifers can best be illustrated through environmental impact. By manipulating rotifer population growth, varied effects can be seen. Preston and Snell (2001) suggested through models that toxicants may alter interactions (such as predation and competition) of species and have predictable yet indirect effects on aquatic communities. Other models, such as Biotic Ligand Models (BLMs), have been created that use data to predict toxicity across phyla,

which is of great interest for risk assessment and the establishment of water quality criteria (De Schamphelaere & Heijerick, et al., 2006). Furthermore, Lapinski and Tunnacliffe (2003) have shown improvement in clarification of municipal wastewater through the addition of rotifers to the ecosystem. Rotifers used in colloid-bound contaminant testing have also revealed problems with the specificity and length of testing in the current system of water toxicity testing (Vignati & Dworak, et al. 2005). In addition to the applications for risk assessment, water quality criteria, and toxicity criteria, the simple ability to alter the growth rate and concentration of a rotifer species by its behavior toward a particular toxicant is extremely powerful; manipulating the rotifer population by behavior could lead to concentrated nourishment for higher trophic levels, such as fish, and eventually lead to a greater number and biomass of fish.

MATERIALS AND METHODS

Fresh cultures of rotifers were initiated twice weekly by adding 50-100 amictic females to a flask containing 250-500mL of the green alga, *Tetraselmis suecica*. Cultures were then aerated, exposed to fluorescent lighting, and maintained at a constant temperature in a 25°C environmental chamber for seven to ten days. Two types of test slides were created for experimentation, a five two-chamber (*Fig. 1*) and a five three-chamber (*Fig. 2*). Cultures were assessed daily for viability by observation of rotifer swimming behavior and the salinity of the culture.

Test Slides

Test slides were created by modifying a pre-printed hydrophobic coating on microscope slides from

Precision Lab. The slides were scraped with a razor blade from two rows of five chambers each into the patterns and dimensions shown in Figures 1&2. A line was then drawn at the median point (9mm and 11mm, respectively) between the connected chambers using a marker on the back of each slide to define the two chambers.

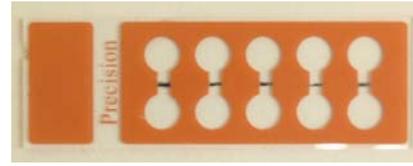


Fig. 1. Example two-chamber test slide. The diameter of each chamber measured 7mm. The channel connecting the test and start chambers measured 4mm long with an average width of 2.5mm.



Fig 2. Example three-chamber test slide. The diameter of each chamber measured 7mm. The channel connecting the test and start chambers measured 8mm long with an average width of 2.3mm.

Preparing Rotifers for Testing

Two-Chamber tests:

Rotifers were isolated from the *Tetraselmis* medium within two hours of the test procedure by pipetting the test animals from the culture to a dish containing artificial seawater (ASW) with matching salinity.

Three-Chamber tests:

Rotifers were hatched in 15 ppt ASW in a 25mL petri dish under fluorescent lighting 24-48 hours before testing. Viability was then assessed based on the swimming speed of females, and only those swimming normally were chosen for experimentation. These rotifers were then isolated from the hatching medium within two hours of the test

procedure by pipetting the test animals from the culture into a Petri dish containing ASW adjusted to the same salinity as the hatching medium.

Loading Rotifers onto the Test Slide

A test slide was placed on the bottom half of an inverted 100 x 15mm polystyrene Petri dish to minimize heating from the microscope light. Using a micropipette, 6 rotifers in 50 μ L of artificial seawater were pipetted into the start chamber of the test slide while on stereoscope, at 10x magnification and a light intensity of 550 lux, with dark field illumination.

Test Procedure

Two-Chamber tests

After the addition of the rotifers, 50 μ L of artificial seawater with matching salinity was pipetted into the opposite well (test chamber) of test slide. Then, using another 50 μ L of ASW, the two drops were joined. Two trials were set up and observed simultaneously.

The illumination on the stereoscope was switched off, and the slide and petri dish were covered with a cardboard box (15.5cm x 9.1cm x 4.2cm) to reduce ambient lighting and air currents. At three and five minutes, the cover was removed, dark field illumination was restored, and the number of rotifers in each test chamber was counted. The box and illumination were restored following the counting of the rotifers at the three-

minute mark. A total of 16 trials with a total sample of 96 rotifers were completed for each test.

Three-chamber tests

After the addition of the rotifers, 50 μ L of artificial seawater with matching salinity was pipetted into both the left and right chambers of test slide. Another 50 μ L of ASW was used to fill each channel connecting the start chamber to the two adjacent chambers. Two trials were set up and observed simultaneously.

The illumination on the stereoscope was switched off, and the slide and petri dish were covered with a cardboard box (15.5cm x 9.1cm x 4.2cm) to reduce ambient lighting and air currents. At 15 minutes, the cover was removed, dark field illumination restored, and the number of rotifers in each chamber was counted. A total of 96 rotifers were observed and were divided into 16 trials with six rotifers each.

Modifications for Tests

Two-Chamber tests

Blank Control

Tests were done with rotifers starting in the top chamber for eight trials and the bottom chamber for eight trials. The distribution of rotifers after three and five minutes was recorded to check for start chamber bias in the test slides.

Light and Dark Tests

Half of the inverted petri dish was covered by two layers of duct tape to produce a light and dark test chamber (Fig. 3). The uncovered side of the dish allowed 550 lux of light to penetrate whereas the side covered with duct tape only allowed 86 lux, an 85% reduction in light intensity. The test chambers were positioned on the petri dish so that only one test chamber was exposed to light (Fig. 4). The rotifers started in the light chamber for 16 trials of this test, and for another 16 trials rotifers started in the dark chamber.

Tests with Rotifers

Specific rotifer groups (e.g., male, non-ovigerous amictic females) were pipetted into 1.7mL Eppendorf tubes and placed into a -80°C freezer to kill them. Prior to use, the samples were allowed to reach room temperature and three freeze-killed rotifers in 50µL of ASW were pipetted into the slide test chamber.



Fig. 3. Petri dish used during phototaxis tests. Half of the dish was covered with 2 layers of duct tape

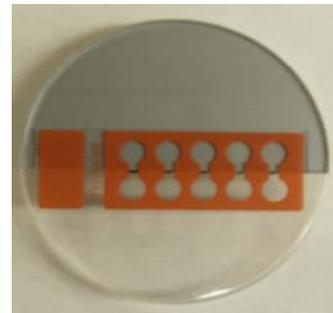


Fig. 4. Placement of test slide on petri dish so that the median line aligned with the edge of the tape.

Tests were initiated with the test organisms and treatment organisms in the same chamber. For these tests, 6 test animals in 30µL of ASW were pipetted into the start

chamber. Three freeze-killed rotifers in 20 μ L were then loaded into the same chamber for a total volume of 50 μ L in the chamber. Fifty microliters of artificial seawater with matching salinity was pipetted into the opposite chamber of the test slide and adjoined with another 50 μ L of ASW added in two 25 μ L drops.

Tests with Algae and Conditioned Medium

Tetraselmis suecica was collected into 1.7mL Eppendorf tubes and frozen at -80°C to kill the cells. After thawing, a cell count was performed with a CELL-VU® hemacytometer to determine cell density. After the initial set up of the test slide, the *Tetraselmis* suspension was vortexed for approximately five seconds, and then 1 μ L of the sample was carefully pipetted into the center of the test chamber.

Conditioned medium was collected by vacuum-filtering a 7- to 10-day-old rotifer culture with a 1 μ m glass fiber filter to remove rotifers and algae. One microliter of conditioned medium was then loaded into the test slide with the same methods used in loading algae.

Three-chamber: Right-chamber tests

Tests with Conditioned Medium

Conditioned medium was collected by vacuum-filtering a 7- to 10-day-old rotifer culture in a 250 ml flask with a one micrometer glass fiber filter to remove rotifers and algae. To minimize mixing between chambers, one microliter of conditioned medium was loaded into the right chamber after 49 μ L ASW was placed in the chamber.

Tests with Mercury, Copper, Selenium, Cadmium, Lead, Pentachlorophenol, Flutamide, and Progesterone

The concentrations (in $\mu\text{g/L}$) of mercury ($0.2\mu\text{g/L}$), copper ($2\mu\text{g/L}$), selenium ($2\mu\text{g/L}$), lead ($8\mu\text{g/L}$), cadmium ($2\mu\text{g/L}$), pentachlorophenol ($2\mu\text{g/L}$), flutamide ($8\mu\text{g/L}$), and progesterone ($8\mu\text{g/L}$) were made by adjustments for percent composition and then serial dilution to the correct stock. Then one microliter of the solution was added to $49\mu\text{L}$ ASW in the right chamber to create the desired final concentration.

Dose Response in Three-chamber Slide: Right Chamber Tests

For the tests with significant results (conditioned medium, copper, selenium, mercury), a dose response at 0.25X, 0.5X, 1X, 2X, and 4X the initial test concentration were conducted. Each concentration test was replicated in 10 trials times with each trial using 6 rotifers and lasting 15 minutes per trial.

Comparison of Dose Response in Start Chamber to Dose Response of Three-Chamber:

Right Chamber Tests

For the tests with significant results (conditioned medium, copper, selenium, mercury), a dose response at 0.25X, 0.5X, 1X, 2X, and 4X the initial test concentration were conducted. Each concentration test was replicated in 10 trials times with each trial using 6 rotifers and lasting 15 minutes per trial.

RESULTS

Figure 5 depicts the effects of treatments on the distribution of amictic females in two-chamber experiments at three and five minutes. Blank tests in the top and bottom chambers showed a slight preference for the top chamber, so in all subsequent tests, the chamber in which the stimulant was placed alternated equally between top and bottom chambers. After using this method, the null hypothesis was that equal distribution would be present between the top and bottom chambers. Significant results were found, however, for taxis to light chambers at three and five minutes (X^2 , $P < 0.001$ —both three and five minute tests) as well as avoidance from dark chamber tests (X^2 , $P < 0.001$ —both three and five minute tests). Tests with conditioned medium showed significant taxis at both three and five minutes (X^2 ; $P < 0.001$ for three minutes, $P = 0.014$ for five minutes). Females with live males showed significant avoidance distribution in the two-chamber test at three and five minutes (X^2 , $P = 0.041$ for three minutes and $P = 0.103$ for five minutes); amictic females with other amictic females also showed avoidance (X^2 , $P = 0.025$ for three minutes and $P = 0.041$ for five minutes). Males showed significant movement towards the freeze-killed females at both three and five minutes (X^2 , $P < 0.001$ for three minutes, $P = 0.0043$ for five minutes). Amictic females also showed significant taxis to the freeze-killed females at both three and five minutes (X^2 , $P = 0.2207$ for three minutes, $P = 0.2207$ for five minutes).

Figure 6 depicts amictic female distribution under control conditions at 3, 5, 10, and 15 minutes. The figure shows the distribution levels from three to fifteen minutes become fully randomized at the fifteen minute mark.

Figure 7 depicts amictic female distribution in left, start, and right chambers in response to conditioned medium in the right chamber after 15 minutes. The control shows random distribution as opposed to the conditioned medium, which shows significant increased taxis toward stimulus in right chamber (ANOVA Oneway, $P=0.008$), random distribution in the center chamber, and significantly lower taxis toward the left chamber farthest from the stimulus. However, conditioned medium tests showed a lack of repeatability in subsequent tests with conditioned medium present in the start (center) chamber.

Figure 8 depicts amictic female distribution in left, start, and right chambers in response to lead ($8 \mu\text{g/L}$) in the right chamber after 15 minutes. This control shows random distribution, whereas the presence of lead shows avoidance with significantly fewer rotifers present in right chamber with the lead than predicted by random distribution (ANOVA Oneway, $P=0.0081$), however this was not replicable in start chamber tests (ANOVA Oneway, $P=0.399$).

Figure 9 depicts amictic female distribution in left, start, and right chambers in response to copper in start chamber at 15 minutes. There is an overall significant trend of avoiding copper in the start chamber (ANOVA Oneway, $P=0.002$); however, no avoidance is seen at the $6 \mu\text{g/L}$ concentration.

Figure 10 depicts amictic female distribution in left, start, and right chambers in response to mercury exposure in start chamber at 15 minutes. There is an overall significant trend of avoiding the toxicant in the start chamber (ANOVA Oneway, $P=0.035$); however, no avoidance is seen at the $0.3 \mu\text{g/L}$ concentration.

For two-chamber tests, no consistent significant distributional effects were observed for *T. suecica* (X2, $0.02 < P < 0.05$ for 3 minutes ; $P > 0.5$ for 5 minutes). For three-chamber tests, no significant distributional effects were observed for cadmium ($2\mu\text{g/L}$), pentachlorophenol ($2\mu\text{g/L}$), flutamide ($8\mu\text{g/L}$), progesterone ($8\mu\text{g/L}$), nor selenium ($4\mu\text{g/L}$).

DISCUSSION

The methods developed are valid ways of measuring rotifer behavior, given the criteria above. These methods can be extended to measuring behavioral responses for many variables, including common run-off chemicals and environmental hormones, as well as rotifer predator-prey interactions. Each time a test is developed, however, the time of response and human measuring time needs to be carefully evaluated so that responses have enough time to take place and be recorded. Also, the concentration of algae and proteins, unlike inorganic substances, needs to be monitored closely for degradation and constant concentration throughout the test in order to preserve validity. These measures are well worth the work to be able to have more specific data in a time scale that is less than one-fourth that of other ecotoxicology methods.

Avoidance often occurs at levels below published lethal concentrations (LC50s) on which many water quality criteria are based. Significant avoidance of dark chambers (and taxis towards light), but the absence of a response towards algal distribution agrees with studies claiming positive phototaxis in rotifers, but interestingly isolates the effects of algae and light to show that rotifers display taxis to light alone, not algae in light.

Amictic females were observed to avoid certain concentrations of lead and selenium, but these tests lacked reproducibility where the rotifers were directly exposed to the toxicants. This may suggest that the impairment of sensory systems occurs quickly by way of these elements or that rotifers, once given the choice between a toxic and nontoxic environment, retreat to the nontoxic environment. If they are never able to move and aware of the nontoxic environment, they may just stay in the center chamber, regardless of the toxicity.

There are many possible explanations for the overall significance of copper and mercury through all tests. Most probable would be the pre-exposure of past generations of the rotifers has caused a more sensitive response to these elements. Enesco et. al (1989) concluded in similar rotifer species that “copper excess enhances lipid peroxidation by the generation of free radicals,” which may be another explanation for the effects seen with copper in this study and the shortened lifespan effects shown in across several copper studies. Finally, Cochrane et. al (1991) showed that copper effects stress protein abundance, which may also be an explanation for these results, but this study offers no significant data to explain the effect of mercury.

Studies across organisms have looked at the various effects of mercury on the environment; these findings can help determine an argument for the avoidance effect seen with mercury. Ionic and elemental mercury occur in nature and have “not yet been characterized as essential for any biologic reaction” (Bidlack, 1998). However, mercury is readily accumulated and has a long half-life in biological tissues, and thusly, defense mechanisms against the effects of mercury are seen. “Based on sulphhydryl binding inside the cell, mercury is trapped [many organisms] to minimize its general distribution

(Bidlack, 1998)” and its effects on essential biologic processes; these effects include depressed nervous system function, hypersensitivity reactions, and systemic autoimmunity, as well as strong effects in nucleic acids and proteins by interacting strongly with the N-binding sites of purines and pyrimidines (Bidlack, 1998)”. Based on these reasons, it is possible that rotifer sensing mechanisms are immediately impaired by mercury. It is also possible that, because of mercury’s strong effects on nucleic acids and proteins, a true defense mechanism against mercury in the population is can be inherited by subsequent generations, unlike other metals in this study.

In addition to avoiding copper and mercury, amictic females avoided other rotifers in some unique cases. Amictic females were also seen to avoid other amictic females and live males, which suggest the population may be able to self-regulate during amictic cycles by staying as dispersed as possible to keep the concentration of the mixis induction protein low for the longest period of time possible. In addition to these findings, live males were shown to display taxis toward freeze-killed females, reconfirming data that chemical senses are how the rotifer sexes sense one-another. Significant taxis toward conditioned medium was also shown, but the effects were not reproducible. This lack of reproducibility suggests that the mixis induction protein degrades quickly and must be in high enough concentrations for rotifers to display any behavioral response, even if it is before the change from mictic to amictic. Interestingly, while live amictic females avoided one another, no behavioral response to freeze-killed females was seen.

Despite finding no significant distributional effects for *T. suecica*, cadmium, progesterone, nor selenium, the effects of other metals and possible rotifer taxis should be

investigated. In addition, though pentochlorophenol and flutamide showed no significant results, the effects of other hormones on rotifer behavior are still unknown.

FIGURES

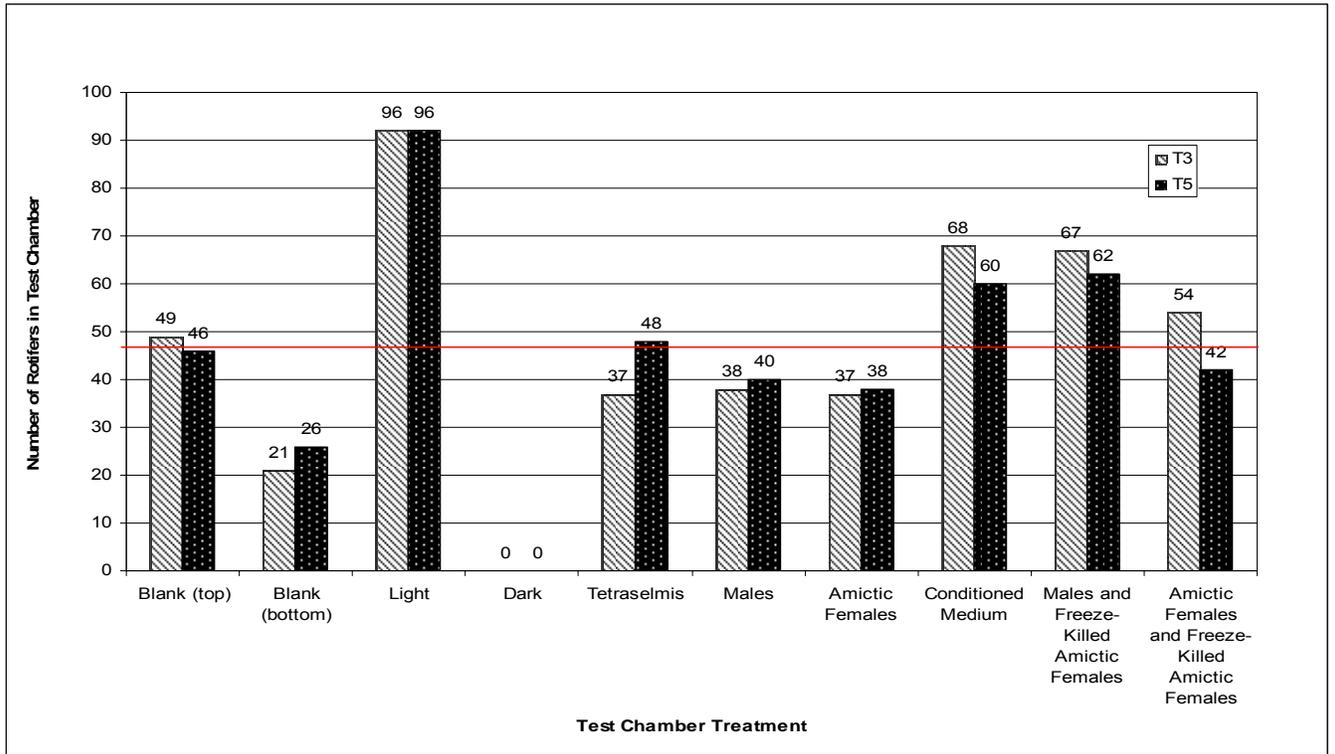


Figure 5 Effect of treatments on the distribution of amictic females in two-chamber experiments at 3 (T3) and 5 (T5) minutes. The horizontal line indicates equal distribution of amictic females in both the test chamber and the blank chamber. Numbers above columns indicate the number of rotifers in the test chamber of the 96 replicates.

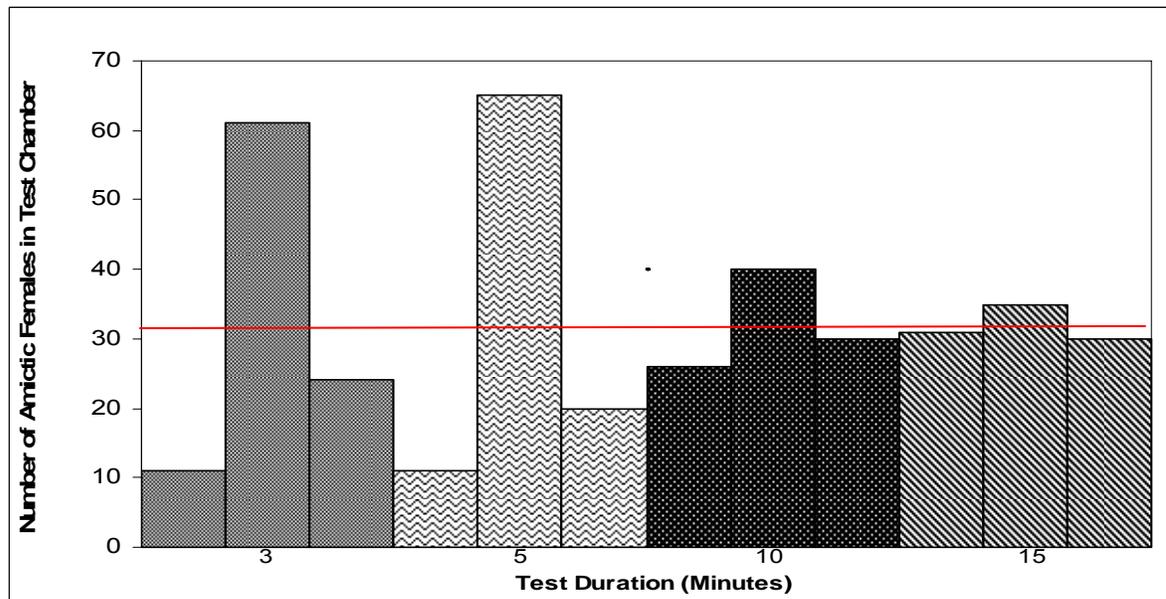


Figure 6 Amictic female distribution under control conditions at 3, 5, 10, and 15 minutes.

Each set of three columns denotes distribution in number of rotifers in the left, center, and right chambers, respectively, at time t. The horizontal line indicates equal distribution of amictic females in all three chambers.

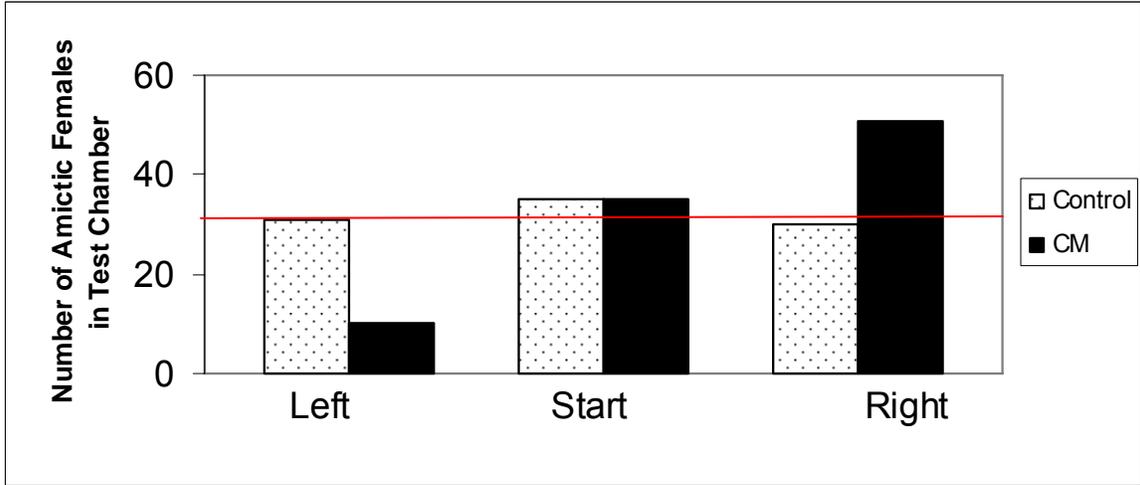


Figure 7 Amictic female distribution in left, start, and right chambers in response to conditioned medium in the right chamber after 15 minutes. Dark columns indicate the number of rotifers in conditioned medium, and light columns indicate the number in the control group. The horizontal line indicates equal distribution of amictic females in all three chambers.

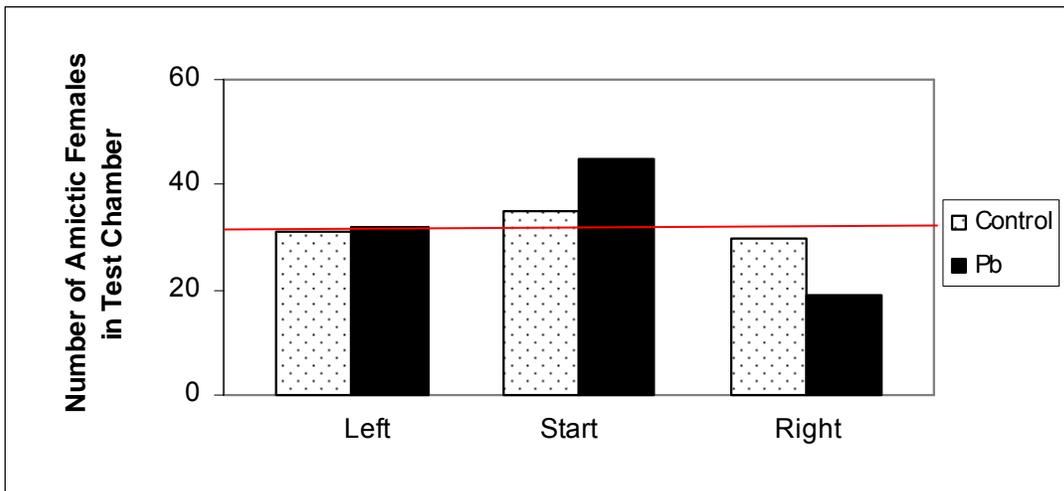


Figure 8 Amictic female distribution in left, start, and right chambers in response to the presence of lead (8 $\mu\text{g/L}$) in the right chamber after 15 minutes. Dark columns indicate the number of rotifers in the chamber with lead, and light columns indicate the number in

the control group. The horizontal line indicates equal distribution of amictic females in all three chambers.

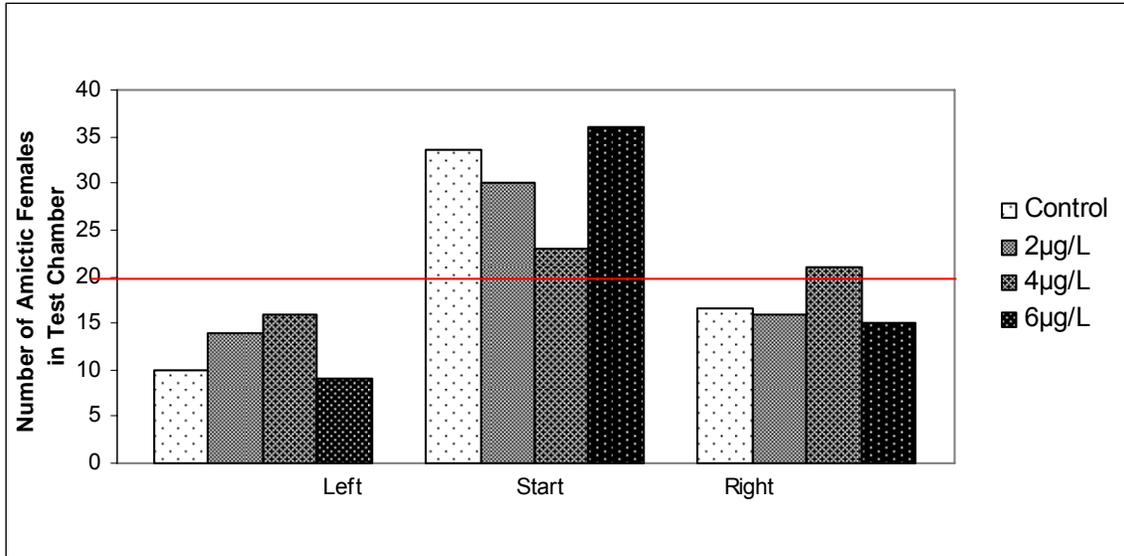


Figure 9 Amictic female distribution in left, start, and right chambers in response to copper exposure in start chamber at 15 minutes. The horizontal line indicates equal distribution of amictic females in all three chambers.

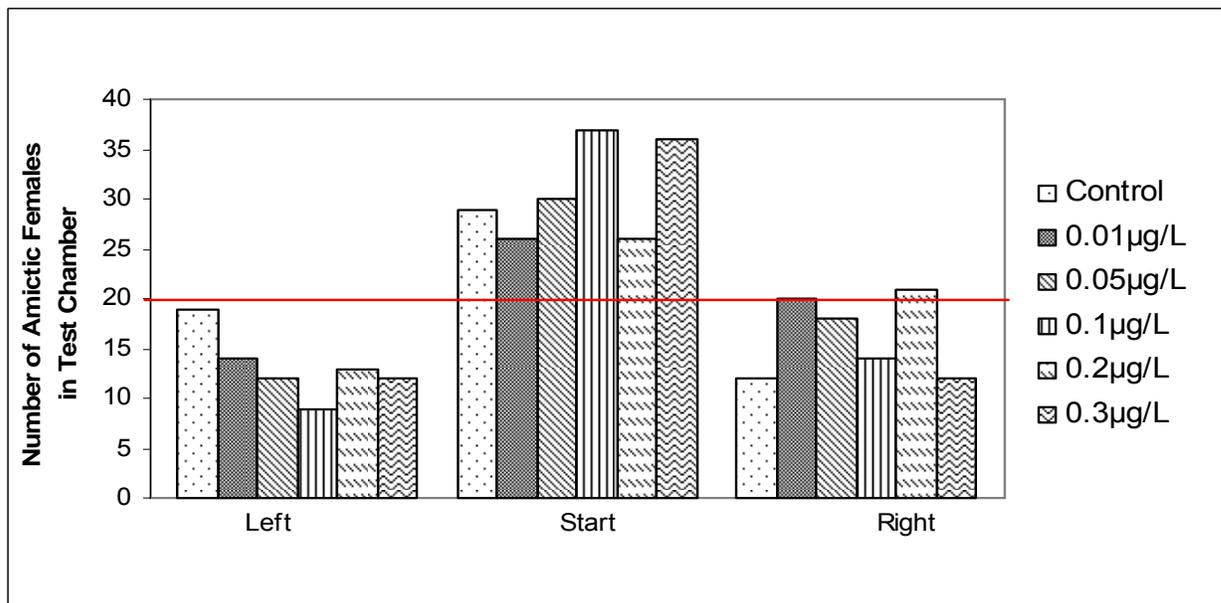


Figure 10 Amictic female distribution in left, start, and right chambers in response to mercury exposure in start chamber at 15 minutes. The horizontal line indicates equal distribution of amictic females in all three chambers.

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