An *in vivo* Multi-Generation Propagation Assay for Endocrine Disrupting Chemicals in the Fruit Fly *Drosophila melanogaster*: Reproductive Functions Disrupted by 4-Nonylphenol

Miki SHIMOHIGASHI[1),*], Kuninori KAIBE[1)], Erika NISHI[1)], Takayuki SAWADA[1)], and Yasuyuki SHIMOHIGASHI[2),3)]

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Abstract

The fruit fly *Drosophila melanogaster* provides one of the best alternatives to the high-throughput use of mammals to screen environmental contaminants. In the present study, we used *Drosophila* to develop a novel *in vivo* multi-generation propagation assay to measure the influence of environmental contaminants on fly development and the reproductive capabilities of female and male flies. Adult *Drosophila* were first conditioned to maintain a stable population affording steady oviposition, by selecting for the next generation only those flies that hatched from third-day eggs. We tested 4-nonylphenol by mixing these with conventional culture medium made of dry yeast. To evaluate the intrinsic effects of this chemical on the reproductive capabilities, the optimal female-to-male ratio for female flies was determined to be 10:10, and 10:1 for male flies. Exposure to 4-nonylphenol in adult stage increased the oviposition, but that in larval stage decreased it. The effect of exposure in larval stage was characterized by increased reproductive function of male especially in the lower chemical concentration after the lapse of three successive generations. These results demonstrate certainly the usefulness of this toxicity test in multi-generation propagation for *Drosophila*.

1. Introduction

There is currently much debate over the health risk associated with the endocrine activity of many chemicals that are either present as contaminants in the environment or used industrially [1, 2]. As a result, an urgent need has been recognized to establish validated screening methods to assay the effects on mammalian hormonal activities of such so-called endocrine disrupting chemicals (EDCs). It is particularly important to develop an *in vivo* assay system to examine the effects of chemicals over several generations. This requirement is particularly demanding, given the typical inter-generational intervals of most laboratory mammals that might be used in such an assay.

So far, there have been three major *in vivo* assay methods for EDC candidates: the uterotrophic assay to test for estrogenicity [3]; the Hershberger bioassay to detect androgens [4, 5]; and the 28-day repeated dose oral toxicity test to examine toxicity [6, 7]. In these assays, mostly mice or rats are utilized to determine, either by direct observation or from tissue weight, the effects of chemical administrations. However, these methods are unlikely to detect genetic influences over multiple generations simply by comparing normal animals with those to whom chemicals have been administered.

The fruit fly *Drosophila melanogaster* is a foremost model species, one that has been used widely as an alternative to mammals in tests of genetic function. *Drosophila* has a set of nuclear receptors ($n = 18$) [8], which may have arisen as orthologs of the more numerous human nuclear receptors ($n = 48$) [9, 10]. Thus, *Drosophila* has been recognized as an excellent alternative model to mammalian species in which to examine the effects of EDCs. An assay method using the fruit fly *Drosophila* would be of fundamental importance as a means to assess the hormonal activity of chemical contaminants, because this

1) Division of Biology, Faculty of Science, Fukuoka University, Fukuoka 814-0180, Japan,
2) Laboratory of Structure-Function Biochemistry, Department of Chemistry, Faculty of Sciences, and
3) Risk Science Research Center, Kyushu University, Fukuoka 812-8581, Japan
* Address correspondences: Correspondence should be addressed to Miki Shimohigashi, miki@fukuoka-u.ac.jp
Abbreviations: EDC, endocrine disrupting chemicals; G0, the parental generation; G1, the first filial generation; G2, the second filial generation

species is easy to rear, has a short life cycle, and can be raised in large numbers well suited to high-throughput studies.

In the present study, we have attempted to develop a novel assay system in which we evaluate *Drosophila* ovulation as an essential effecter of endocrine hormonal activity in the presence of candidate environmental chemicals, especially to assess *in vivo* those effects that are manifest over several successive generations. Our first attempt was to establish a procedure to assess the toxicity of chemicals in multi-generation propagation. As a model chemical contaminant, we have chosen here 4-nonylphenol, which is implicated in endocrine disruptor activity. It should be noted that 4-nonylphenol was one of the earliest EDCs acknowledged as a xenoestrogen for the mammalian endogenous estrogen 17β-estradiol, while 17β-estradiol itself has also been acknowledged as one of the most widespread EDCs [11, 12]. This is due to weak ability of 4-nonylphenol to mimic estrogen and in turn disrupt the hormonal control of homeostasis in affected organisms.

2. Materials and Methods

2.1. Materials

4-nonylphenol, CAS no. 84852-15-3, Technical grade, was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol (EtOH) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Agarose, sugar, corn powder, and dry yeast were obtained from Ina Food Industry Co., Ltd. (Nagano), Dai-Nippon Meiji Sugar Co., Ltd. (Tokyo), Sunny Maids Co., Ltd. (Shizuoka), and Mitsubishi Tanabe Pharma Co., Ltd. (Osaka), respectively.

2.2. Animals and culture medium with/without test chemicals

For dietary investigations of *Drosophila melanogaster*, the wild-type strain Canton S was selected, because it has a steady rate of oviposition. Flies were reared in medium with a composition given below, and maintained at 25˚C in an L12:D12 light:dark cycle. For preparation of the culture medium, agarose (8 g), sugar (100 g), corn powder (40 g), and dry yeast (60 g) were first mixed in water (1,000 ml), and the resulting mixture was boiled for 20 min, and then cooled down to 50-60˚C. To this mixture were added the antiseptics 5% *p*-butylbenzoic acid/70% EtOH (5.3 ml) and propionic acid (2 ml), and the antibiotics penicillin (6.67 unit) and streptomycin (16.67 unit). Test compounds, either 4-nonylphenol (0.022 mg, 2.20 mg, and 220 mg, for 1×10^{-7} M, 1×10^{-5} M, and 1×10^{-3} M final concentrations, respectively) was dissolved in EtOH (10 ml) and added to the medium to make up a final volume of 1,000 ml. Approximately 24 ml of the resulting dietary medium was poured into a sterile plastic dish 92 mm in diameter, and thus each preparation was just sufficient to run a few assays for 2-4 days with about 40 plates. 4-Nonylphenol was judged to be stable, since all of the constituents in the medium are chemically inert for this compound.

2.3. Test flies for multi-generation propagation

In order to select flies for the multi-generation propagation assay, ten female *Drosophila* flies were mated with the same number of male flies in a dish of normal medium. The number of eggs was counted everyday, and it was found that a quite large number of eggs were oviposed in a day from the third to fifth days and also on the eighth day. Compared the daily numbers of eggs laid between third and eighth day after mating within two groups of flies hatched from the third and eighth days of eggs, the number of eggs laid from the flies hatched from the third days eggs was more stable than those of flies hatched from the eighth days eggs (data not shown). Therefore, we chose the flies hatched from the third day eggs as the test flies for multi-generation propagation.

2.4. Number of flies mating

To assess reproductive risk based on the exposure to chemicals, the number of laid eggs and adult flies that emerged from those eggs were taken to reflect respectively the reproductive functions of the female or male of the parental generation. To uncover such effects, the optimum numbers of male and female flies engaged in mating were first determined. In particular, to determine an appropriate male-to-female ratio reflecting the female's reproductive function, three different ratios were examined, a male: female ratio of 10:10, 2:10, or 10:2. The number of eggs laid was counted separately each day in all cases. Furthermore, to determine the appropriate number of female flies to have the opportunity to mate with a fixed number of male flies, reflecting the reproductive function of the males, we examined five combinations, 5, 10, 15, 20 or 25 females when put with 2 males, to count the eggs laid and the adult flies that emerged.

2.5. Assay method for reproduction

In order to establish the method to obtain the fly group for a multi-generation assay, we used the flies that

emerged from the third-day eggs of the parental generation. In the present study, from consecutive generations obtained from such a generalized method, the flies of, for example, the tenth generation was used for the first experimental generation. This parental G0 generation was reared from larval stages held on a normal medium. After their eclosion, the appropriate numbers of males and virgin females were put together in a dish on a selected medium (see 2.1.) (Figure 1). In order to evaluate female reproduction, these flies were transferred everyday to a new dish of fresh medium, and the number of eggs laid

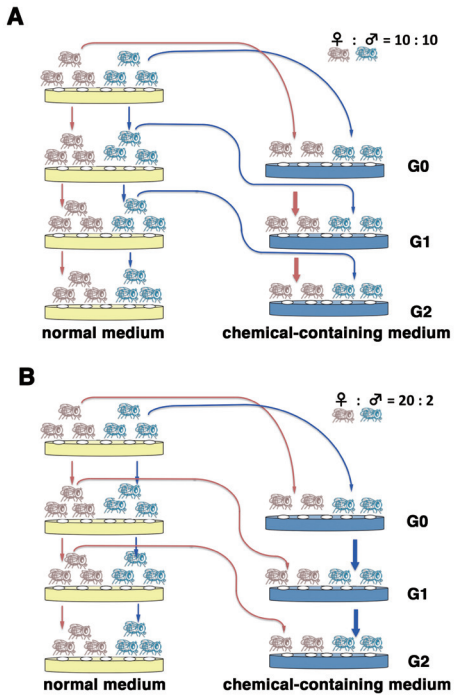

Figure 1: Schematic representation of *Drosophila* multi-generation propagation assays to test female (A) and male (B) reproduction profiles. The parental generation (G0) was reared in its larval stages on a normal medium (yellow dish). After eclosion, the appropriate numbers of males and virgin females were put together in a dish having a selected medium (blue dish). (A) To assess the reproduction of females, 10 female flies that emerged from eggs laid by the parental generation on the third day were mated with 10 males that had grown up on the normal medium (see the results shown in Figure 2). The flies were transferred everyday to a new dish of fresh medium to count the number of eggs laid. G1 and G2 female flies were raised from their larval stages in the medium containing a chemical. (B) To assess the reproduction of males, 2 male flies that emerged from eggs laid by the parental generation on the third day were mated with 20 females that had grown up on the normal medium (see the results shown in Figure 3). The flies were then transferred everyday to a new dish of fresh medium, and the numbers flies emerged from the laid eggs were counted as well as the eggs laid. G1 and G2 male flies were raised from the larval stage in the medium containing a chemical.

counted under the dissecting microscope using a cover for the dish ruled with 1 cm squares, to facilitate counts. These counts were continued for four consecutive days. In contrast to such counts to evaluate female reproduction, the number of eggs and adult flies emerging from those dishes was counted to assess male reproduction.

To assess the reproduction of females of the next generation, female flies that emerged from eggs laid by the parental generation on the third day were mated with males that had grown up on the normal medium (Figure 1). The females of the first- and the second-generation descendants (G1, G2) in the assay of female reproductive function were raised from the larval stage in medium containing chemicals. On the other hand, to assess the reproduction of males, male flies that emerged from eggs laid by the parental generation on the third day were mated with females that had grown up on the normal medium (Figure 1). The parental generation (G0) was also reared first in a normal medium and then exposed to the test chemical 4-nonylphenol only after eclosion. The males of the first- and the second-generation descendants (G1, G2) in the assay of male reproductive function were raised from their larval stage in medium containing 4-nonylphenol.

2.6. Data analysis

Statistical analyses were performed using STATISTICA 6.0 software (StatSoft, www.statsoft.com). Differences in the number of eggs and descendants were analyzed using a Student's *t*-test, and the variation the numbers of eggs during eight days for the determination of mating ratios a one-way ANOVA with Tukey's multiple comparison test.

3. Results

3.1. Selection and culture of test flies for multi-generation propagation

For the multi-generation propagation assay, it was important to attain a stable population of *Drosophila* flies, since a great number of eggs were necessary for continuous experimentation. Fruit flies lay eggs everyday after eclosion, although the number of eggs varies considerably from day to day. In a preliminary study, in which ten each of female and male *Drosophila* were bred together in a dish of normal medium, we found a form of stable rhythm in oviposition, which peaked at the third to fifth days and also at the eighth day after eclosion (data not shown).

We further tested the flies hatched from eggs that had been laid on the third and eighth days, in order to examine

whether their oviposition rhythms were flat and stable. Flies hatched from eighth-day eggs exhibited an uneven oviposition rhythm with a peak at the third day. By contrast, flies hatched from third-day eggs showed a fairly steady rate of oviposition with approximately 100 eggs per day during the second to seventh days (data not shown). Thus, we decided to use flies hatched from third-day eggs for the next generation.

In order to select flies that lay eggs at a stable daily rate, starting from the wild-type we raised flies so that the third-day eggs were used for the next generation. As a result, from the third generation onward, the number of eggs laid attained a stable and invariable daily rate (data not shown), and we decided to initiate the experiment by using flies of the tenth generation from such parents. Flies of this generation were designated as "G0" in the multi-generation propagation assay in the remainder of this study.

3.2. The number of mating flies required to reflect the reproductive function of females

Prior to evaluating the effects of chemicals on the reproductive capability or functions of female and male flies, it was important first to evaluate their normal potential for reproduction. In the multi-generation propagation assay, it was necessary to adopt a concrete female-to-male ratio to combine flies that would feed and mate together, and that should reflect the reproductive potential of each sex. In order to decide the number of male and female flies for this match, we monitored the number of eggs and their descendants by making different pairing ratios.

To determine the female-to-male ratio reflecting the reproductive function of female flies, we carried out the experiment using three combinations: i.e., 10:10, 10:2, and 2:10 female-to-male ratios. Ordinary medium was utilized for this series of experiments. The results are shown in Figure 2. The average numbers of eggs laid by ten females kept at either the 10:10 or 10:2 ratio were judged to be almost the same, because there is not significant difference between values at each day by student t-test, and variations during eight days by a one-way ANOVA with Tukey's multiple comparison test. This implies that the rate of oviposition depended only upon the number of female flies present, and not the number of male flies. On the other hand, the rate of oviposition from females held in the 2:10 ratio was only about 20% that of the higher two ratios (Figure 2). We thus concluded that an equal number of male and female flies was sufficient to assess female reproduction.

3.3. The number of mating flies required to reflect the reproductive function of males

Determination of the female-to-male ratio that best reflects the reproductive function of males was achieved by examining the outcomes of five different mating combinations, namely, 5, 10, 15, 20, or 25 female flies mated to 2 male flies. The number of eggs laid on the third day together with the number of adults emerging from these eggs is shown as a histogram (Figure 3). When the ratio of flies increased from 5 to 20 females for 2 male flies, the number of eggs laid increased from about 90 to 400. The egg number in the case of the 25:2 mating ratio was almost the same as that for the 20:2 ratio. The number of emerged adults also increased with the increasing number of females to 10. However, the number of adults reached around 150 within 15 to 25 of females, where there is not significant difference. The number of adult flies was only about 40% of the eggs laid in the tests of combination of 20:2 and 25:2 ratio. Collectively, these findings indicate that the number of emerged flies already attains a maximum with a mating ratio of 15 females to 2 males. Thus, it is evident that a ratio of one male to ten females represents the maximum reproductive capability of parental males.

3.4. Effects on female reproduction of chemical contaminants in the medium

Evaluation of the effects of chemical contaminants on the reproductive capability of female flies was carried out using batches containing the same numbers of females and male flies, as demonstrated above. Ten individuals of each sex raised on the normal medium were mated on medium containing the chemical, as the G0 generation (Figure 1). Flies fed on the control medium showed a steady rate of oviposition on the third and fourth days because they had previously been hatched from third-day eggs (see above).

The numbers of eggs laid by females of the G0 generation that exposed to 1×10^{-7} M 4-nonylphenol increased the rate of oviposition by approximately 20%, compared to the control in G0 generation. On the other hand, those of the G1 generation revealed decreasing by approximately 25%, compared with those of control in the G1 generation (Figure 4A). It should be noted that, although the parental generation (G0) first grew up on normal medium, the first and second filial generations (G1 and

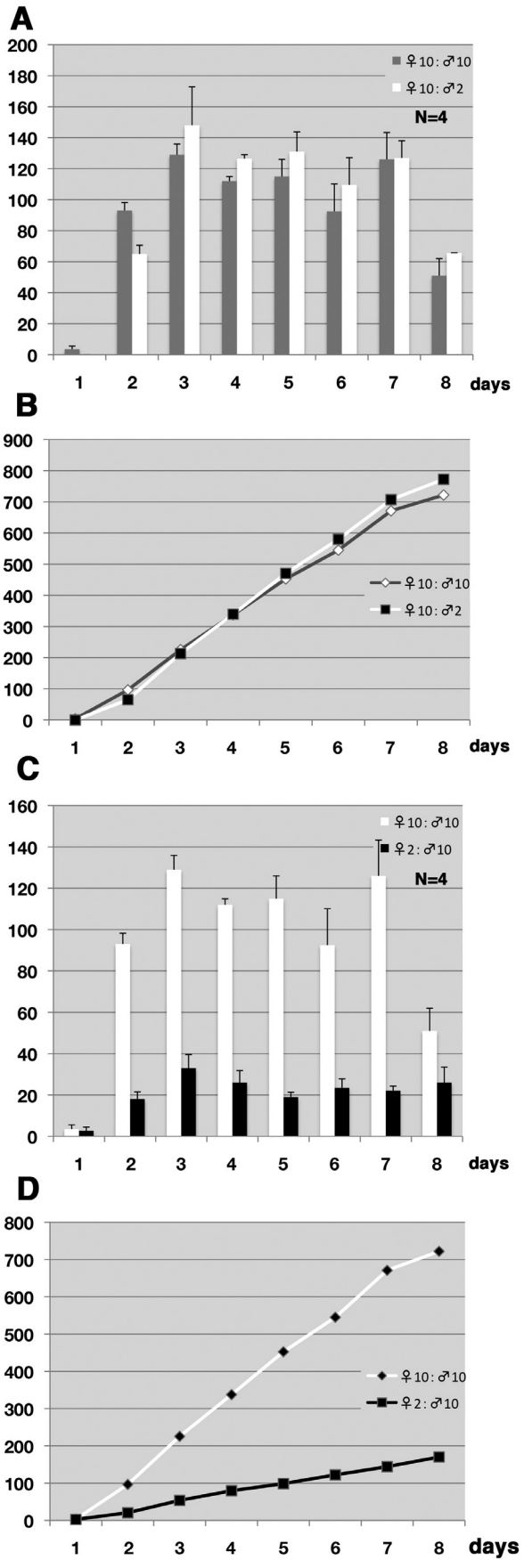

Figure 2: Daily oviposition by female *Drosophila* mated with different numbers of males. This experiment reveals the female-to-male ratio that reflects optimum reproductive function in female flies from three different mating combinations. (A) Daily oviposition of females. 10 females were mated with either 2 or 10 males. Each experiment was repeated at least four times, and the average numbers of laid eggs in the respective test days are shown with a standard error from these independent experiments. (B) Cumulative numbers of laid eggs. The numbers of eggs laid were accumulated cumulatively everyday for 10 females which were mated with either 2 or 10 males (data from panel A). (C) Daily oviposition of females. 2 females were mated with 10 males. Control was carried out with 10 females, mating with 10 males. Each experiment was repeated at least four times and the average numbers of laid eggs of respective test days are shown with a standard error. (D) Cumulative numbers of laid eggs. The numbers of eggs laid were accumulated cumulatively everyday just as for panel B, using the data from panel C.

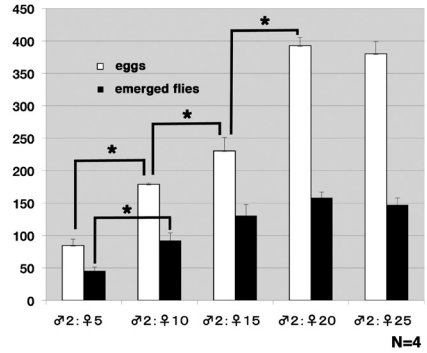

Figure 3: Determination of the optimum mating ratio for testing the reproductive function of male flies. Total numbers of eggs laid are shown together with the numbers of adults emerging from these for the first four days. Each bar shows the average of four samples together with the standard errors. Empty white bars show the numbers of eggs laid, and filled black bars show the numbers of adults emerged. The astarisks indiate the significant difference ($p < 0.01$).

G2, respectively) were raised on medium containing the chemical contaminants. Therefore the effect in G0 is due to the exposure only in the adult stage, and those in G1 and G2 are due to the exposure both in larval and adult stages. These results, therefore, imply that the exposure in adult stage increased the oviposition, and the exposure in larval stage decreased it. The decrease in egg production became slightly further pronounced in the G2 generation at 1×10^{-7} and 1×10^{-5} M 4-nonylphenol, whilst the reverse effect was seen in 1×10^{-3} M 4-nonylphenol (Figure 4B, C). This effect was more in the lower dose of chemical (Figure 4C). On the other hand, the relative oviposition rate was diminished by 1×10^{-3} M 4-nonylphenol in G2 generations.

When the effects on oviposition were evaluated with increased doses of 4-nonylphenol in female *Drosophila*,

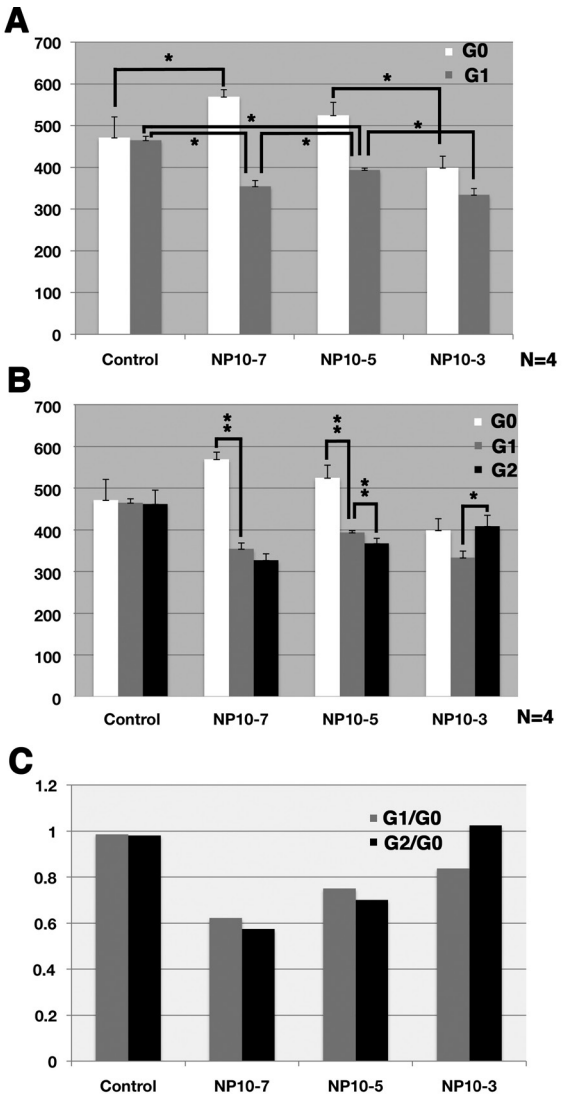

Figure 4: Effects of 4-nonylphenol on oviposition of female *Drosophila* in successive generations. The longitudinal axis in the panels (A) and (B) shows the total number of laid eggs from the first four days, while that in (C) shows the ratio of the total number of laid eggs in the generation tests. In these generation tests, ten female flies were mated to ten males. NP means 4-nonylphenol, and numerals (10-7, 10-5, and 10-3) attached to NP show the concentrations of NP as 1×10^{-7}, 10^{-5}, and 10^{-3} mol, respectively. (A) The generation tests in G0 and G1. Bars show the total ovipositions averaged from four samples together with the standard errors. Empty white bars show the numbers of laid eggs in G0 generation, and gray bars show those in G1 generation. The asterisks indicate the significant difference ($p < 0.01$). (B) The interrelation between three successive generations G0, G1, and G2. Total ovipositions in G2 are shown in black bars. The double and single asterisks indicate the significant difference ($p < 0.01$ and $p < 0.05$, respectively). (C) Comparison of total ovipositions between the generations G0, G1, and G2. The ratios of laid eggs in G1 or G2 over those in G0 represent the effectiveness of chemical exposure in the larval stage.

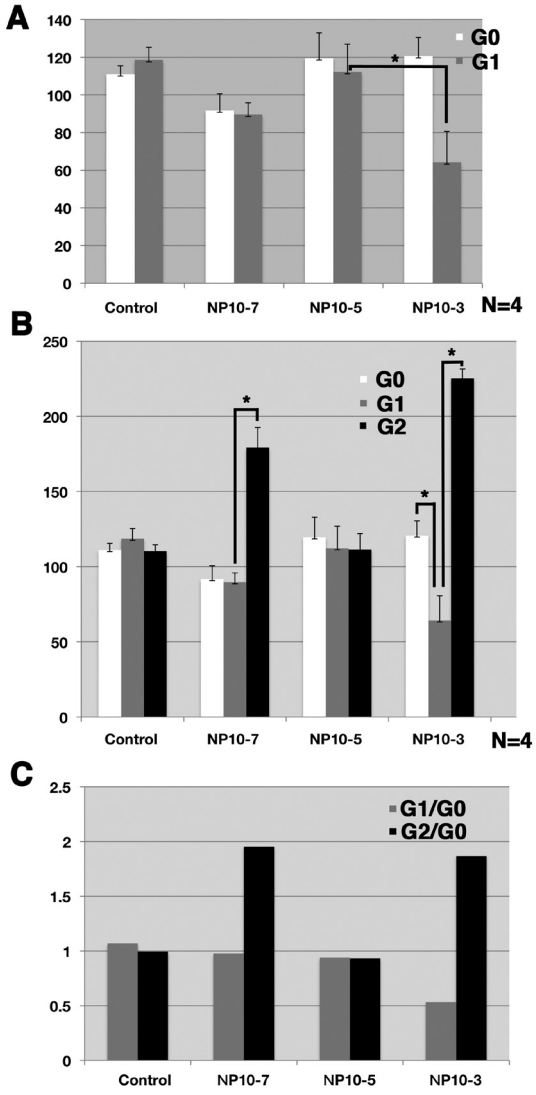

Figure 5: Effects of 4-nonylphenol on the reproductive capability of male *Drosophila* of successive generations. The longitudinal axis in the panels (A) and (B) show the total number of descendants from the first four days, while that in (C) shows the ratio of those descendants in the generation tests. In these generation tests, two males were mated to twenty females, as shown in Figure 1B. NP means 4-nonylphenol, and numerals (10-7, 10-5, and 10-3) attached to NP show the concentrations of NP as 1×10^{-7}, 10^{-5}, and 10^{-3} mol, respectively. The data are the averaged from four samples testing. (A) The number of descendants in G0 and G1 generation test. (B) The interrelation between three successive generations G0, G1, and G2. Total descendants in G2 are shown in black bars. (C) Comparison of the reproductive capability of male *Drosophila* between the generations G0, G1, and G2. The ratio of the numbers of descendants in G1 or G2 over those in G0 generation tests represents the effectiveness of the chemical exposure in the larval stage.

diverse dose-dependencies were observed in G0 generation. In the case of 1×10^{-3} M 4-nonylphenol, the hyperactive effect on oviposition seen at lower nonylphenol was diminished to approximately 90% of the rate to the control G0 generation. This was the reverse effect to that seen with 1×10^{-5} M and 1×10^{-7} 4-nonylphenol, for which the relative to controls of the corresponding rates were increased at these lower concentrations. By contrast, in the G1 generation a suppression of the rate of egg laying was seen in a dose-dependent manner with more severe reductions seen with reduced concentrations of 4-nonylphenol. In the G2 generation, the effect was more severe with prior exposure to 1×10^{-7} and 1×10^{-5} M 4-nonylphenol, and the rate of oviposition recovered by exposure to 1×10^{-3} M 4-nonylphenol (Figure 4C). These results are compatible with 4-nonylphenol exerting two actions, one enhancing and one suppressing on the rate of ovulation, each action having a different dose-dependency.

3.5. Effects of chemical contaminants on male reproduction

In order to examine the effects of chemicals on the reproductive function of male flies, we used the optimal male-to-female mating ratio of 1:10, with 2 male and 20 female flies, and counted the number of adult flies that later emerged. Although there is not seen the significant deference in G0 generation, the number of descendants in G1 was decreased at $1x10^{-3}$ M 4-nonylphenol. This effect was due to the exposure with the chemical in the larval stage (Figure 5). When the effects were evaluated for three successive generations after exposure with the different concentrains, there was not seen the significant different at 1×10^{-5} M 4-nonylphenol. The numbers of descendants from G2 flies were approximately double scored at 1×10^{-7} M and 1×10^{-3} 4-nonylphenol. This means that exposure to 1×10^{-3} M 4-nonylphenol at the larval stage diminished male fecundity, but that exposure of it successively did affect diversely. These results imply that 4-nonylphenol is not effective on the male fecundity when exposed at adult stages, but it is effective repressively at the beginning, and then hyperactively when exposure occurs at higher concentration in larval stages (Figure 5A).

4. Discussion

4.1. Animal selection and preparation for chemical contamination test

Despite its many differences from human morphology and body plan, the fruit fly *Drosophila melanogaster* has a number of compelling qualifications as a model system for high-throughput studies on cellular and developmental processes common to both. Thus, *Drosophila*, long recognized as one of the most investigated species in classical and molecular genetics, is now widely studied using methods adopted from biochemical, cell biological, and physiological approaches, to address problems requiring a multidisciplinary approach, such as developmental and reproductive biology [13], as is clear from studies on the genetics and developmental biology of nuclear receptors [8].

Two clear qualifications that recommend *Drosophila* are, first, that it has a short generation time, approximately 10 days at room temperature. This allows the *in vivo* assay of contaminant chemicals over several generations. Thus, ten generations of *Drosophila* take only about 100 days to pass, whereas it would take about 200 years for the same number of human generations. In this way *Drosophila* provides opportunities for multi-generation studies on the effects of environmental chemicals on hormonal activity that would not be possible on vertebrates. *Drosophila* has a second clear advantage for reproductive studies, which is that it has a high fecundity. Female flies can lay more than 800 eggs in a lifetime. Reliable egg production is brought from the advantages upon which classical genetics are based, that males and female *Drosophila* are readily distinguished and virgin females easily isolated, to facilitate genetic crossing.

To investigate the effects of dietary contaminants, we generated a protocol to ensure that flies first had a predictable egg-laying state. This required that we first select the correct time of egg-laying after emergence, and then that we select the optimum female-to-male ratio. Despite the long history of *Drosophila* culture methods (e.g. [14]), we found no systematic report of these data in the literature, although they were particularly important to evaluate the acute effects of dietary chemicals on their reproductive capability. The stable rate of egg-laying we obtained was of the order of peak rates previously reported [15, 16], as is the decline in this rate with age post-eclosion, but the rates we report for different sex ratios are apparently novel. Thus, the female-to-male ratios reflecting the peak reproductive function were found to differ for female and male flies, being 10:10 and 10:1, respectively.

4.2. Effects of chemical contamination on oviposition and emergence rate

It was previously reported that injecting 17*β*-estradiol into pupae of the silk moth *Bombyx mori* decreased egg production [17]. Similarly, exposure of jute hairy caterpillars (*Spilarctia oblique*) to estrogen has been reported to reduce egg production [18]. To evaluate the effects of chemicals on the reproductive capability of female flies over several generations, we allowed equal numbers of both sexes to mate and found that 4-nonylphenol exhibited parallel effects on oviposition. Since the oviposition of control flies is almost steady on the third and fourth days (data not shown), 4-nonylphenol seems therefore to have a stimulating effect on egg laying that is optimized on the third day. This effect decreases with the lapse of successive generations, however. Female flies in their G0 generation are first exposed to the chemical contaminants only after eclosion, allowing their oocytes to have developed normally. Oocytes of the G1 and G2 generation females are exposed from the larval stage, allowing the chemicals to affect ovarian development and oocyte differentiation starting within the larval stage. The decreased oviposition seen later, in the adult females that emerge from such larvae, must therefore be the outcome of the chemical contaminants added to the medium. Consistent with this interpretation, the decrement was more obvious in the G2 generation than in the G1 generation except in 1×10^{-3} M 4-nonylphenol.

For the effects of chemical contaminants on the reproductive capability of male flies, we tested the best male-to-female mating ratio of 1:10. 4-nonylphenol did not affect the G0 generation. In the G1 and G2 generations, however, these chemicals had different effects, showing no difference for 1×10^{-5} M 4-nonylphenol. This inconsistency indicates that 4-nonylphenol affects the development of reproductive tissues in males in the larval stage, but that 4-nonylphenol may have no such action at the concentration used (1×10^{-5} M). Since the effect of 4-nonylphenol on G2 was reverse as that on G1, 4-nonylphenol appears to have diverse effects. At this moment, the mechanism of them are not known.

4.3. Apparent low-dose effects by chemical contaminations

There was a clear dose-dependence in the effects on the oviposition and fecundity of males fed on 4-nonylphenol (Figures 4 and 5). Comparing exposures to 1×10^{-7} M and 1×10^{-5} M 4-nonylphenol, the relative oviposition rate was higher with 1×10^{-7} M than with 1×10^{-5} M 4-nonylphenol in the G0 generation, which reveals the effect of exposure at the adult stage. On the other hand, reduction of the relative rates of oviposition from that of the G1 and G2 generations shows the effects of exposure to the contaminant at the larval stage. The effect of exposure at the larval stage is also prominent at lower dose rates. Thus, the oviposition was found to increase in response to the lowest dosage of 4-nonylphenol administered. In other words, lower concentrations were more effective than higher concentrations. These are so-called ‘low-dose effects’, which have been reported for example for bisphenol A [19]. Many lines of evidence have shown that exposure of experimental animals to low doses of bisphenol A may cause profound physiological effects.

It was assumed that the low-dose effects might be mediated by signaling pathways that dramatically amplify effects, such that large changes in cell function can occur in response to very low concentrations of contaminant [19, 20]. However, the reported range of ‘low-doses’ of bisphenol A is just compatible with the concentration range with which endogenous hormones interact specifically with their own target receptors. Indeed, the lowest concentration in this study, 1×10^{-7} M, lies within the usual range of drug concentrations that cause the maximal response of these hormone receptors.

4.4. Perspectives for nuclear receptor mediating the effects of chemical contaminations

Our findings from both female and male flies indicate that the effects of the 4-nonylphenol on reproduction are possibly to be mediated through actions on nuclear hormone receptor(s). The *Drosophila* genome encodes 18 nuclear receptors, compared with 48 in humans, providing the smallest complete set of receptors known in any genetic model system. In spite of this small number, fly nuclear receptors represent all major subclasses of human ones, including orthologs of key human receptors [8]. The estrogen-related receptor in *Drosophila*, dERR, is an ortholog of a number of human estrogen- (ERs; ERα and ERβ) and estrogen-related receptors (ERRs; ERRα, ERRβ, and ERRγ) [21]. These human receptors are representative of nine such steroidal nuclear receptors, for which *Drosophila* possesses only the single dERR receptor. It is of course unlikely that the chemicals we have tested affect only dERR, even though this receptor may mediate some of the actions we observe on egg laying, and even though estrogenic compounds would interact mainly

with the estrogen-related receptor. Adding that, the ecdysone receptor EcR also might contributes to reproductive function and may provide an additional target. To distinguish the exact combination of receptor activation for each contaminant chemical, additional *in vitro* receptor assays will however be necessary.

5. Conclusion

In the present study, we have used the fruit fly *Drosophila melanogaster* to establish an *in vivo* multi-generation propagation assay to measure the influences of environmental contaminant chemicals on the reproductive capabilities of female and male flies. This system has considerable promise as a high-throughput assay for a range of environmental contaminants that affect reproductive output and are of probable endocrine origin, as we demonstrate for 4-nonylphenol. In female flies, the exposure to 4-nonylphenol in adult stage enhances, and that in larval stage decreases the oviposition rate. An effect we suspect is due to the estrogen-related receptor. The assay we report is well suited to assess the *in vivo* influence of contaminant chemicals, especially through successive generations. Further evaluation of the molecular targets of such action is facilitated by the genetic methods available in particular in *Drosophila*.

Conflict of Interests

The authors declare that there is no conflict of interest.

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