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Preliminary Study on Zebra Fish's DNA Exposure to Di-2-ethylhexyl Phthalate Effects

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ABSTRACT

Di-2-ethylhexyl phthalate may interfere with the normal endocrine function of humans and other animals, affecting their growth, development, behavior and reproductive ability. This study examined the effects of environmental factors, such as temperature, pH and dibutyl phthalate (2-ethylhexyl) phthalate concentration, on the breeding by zebra fish. Gene confirmation was used to determine the effect of phthalate (2-ethyhexy) phthalate *in vivo* on the growth, development, behavior and reproductive ability of zebra fish.

Keywords: Di-2-ethylhexyl phthalate; Zebra fish; DNA; DEHP

1. INTRODUCTION

Phthalate (2-ethylhexyl) phthalate (di-2-ethyl-hexyl phthalate, DEHP) is used in many chemical processes as a UTV plastic additive (Table 1). Dibutyl phthalate (2 - ethylhexyl) phthalate is considered to be likely to interfere with the normal endocrine function of humans and other living organisms. The chemicals that enter the body of an animal can affect the animal's endocrine system in a way that is similar to the effects on hormones, or interfere with the balance of the endocrine system and its function (Hicks et al., 1980; Liu et al., 2000).

1.1 Objective

The national prevalence of zebra fish was studied. The effects of environmental factors, such as temperature, pH, dibutyl phthalate (2-ethylhexyl) phthalate concentration, on breeding by zebra fish were considered. The breeding environment of zebra fish was not contaminated. The favorable temperature and pH of water were maintained for zebra fish. An experimental treatment group is compared with a control group. Changes in the DNA gene caused by DEHP were confirmed. The effects of phthalate (2 - ethylhexyl) phthalate *in vivo* on the growth, development, behavior and reproductive capacity of zebra fish were examined.

1.2 Phthalate (2 - ethylhexyl) Phthalate

Phthalate (2 - ethylhexyl) phthalate, an environmental hormone, has been banned (Gray et al., 2000). Reports of a higher incidence of urogenital anomalies of newborn animals, such as crytorchidism, hypospadias, and reproductive abnormalities following exposure to high levels of DEHP chemicals in the environment, have led to public concern that these agents may harm human reproductive health (Sharpe et al., 2000). The researcher (CSTEE, 2002) concluded that DEHP (can OR has the potential to) adversely affect the reproductive capacity of humans. Indeed, several of the

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proposed mechanisms by which this agent influences testicular function in rats and mice are relevant to humans. The Japanese Environment Agency has listed the plasticizer dioctyl adipate (di-2-ethylhexyl adipate, or DOA) as an environmental hormone. An International Environmental agency, United States and Canada have banned the oral use of DEHP and its use in toys in a way that could result in direct contact with the chemicals. The E.U. also prohibits the manufacture of toys that contain) DEHP for children who are younger than three years old. The US EPA limits for DEHP in drinking water is 6 ppb. The U.S. agency OSHA's limit for occupational exposure is 5 mg/m^3 of air.

1.3 Biological and Toxic Materials

The toxicity of DEHP to various animals varies greatly. Rats and mice are the most sensitive to the chemicals, followed by hamsters and guinea pigs. Feeding a high dose of DEHP to marmosets (2500 mg/kg/day) for 13 weeks had no effect on the liver. A 104-week carcinogenicity study of rats and mice revealed liver cell adenoma and cancer following ingestion of DEHP. Available data are very limited.

1.4 Mutation Test (Mutagenicity Study)

Tests on rats have revealed no clastogenic effect of DEHP. Chinese hamster ovary (CHO) did not exhibit mutagenicity in response to Salmonella typhimurium strains and DEHP that are commonly used in mutation tests. In Zeyi mouse rat chronic toxicity test (rats), the mice were fed for 21-day food with DEHP concentration of 0.01~2.5% in toxicity test. The body weight and liver weight were significantly changed, and the size of the male rat liver and the level of cytoplasmic basophilia decreased (Gray et al., 2000, Kim et al., 2002). Pregnant females who were fed 1, 250, 750, and 1000 mg/kg.d produced offspring with significantly reduced body weight. Experiments verified that DEHP affects the growth of offspring or causes premature birth, and detrimentally affects the reproductive capacity of the female or the offspring (Ding et al., 2010).

1.5 Effects of Asthma Human

Research has verified that DEHP can induce allergic symptoms, including human asthma. Bornehag et al. (2004) surveyed 198 patients with allergic symptoms and 202 healthy children and found that DEHP caused asthma and other allergic symptoms. As the number of allergens environment has been declining, the number of sufferers of asthma has been increasing. Nowak et al. (2004) noted that other man-made chemicals and continuously increasing concentrations of air pollutants may be responsible the growth of pathogenic factors of asthma (Gi et al., 2006).

Table 1 Potential source of exposure and health effects of selected phthalates

Phthalate diester	Potential sources of exposure	Potential health effects
Diethy1 phthalate	Coating,dyes,insecticides	Reduced growth rate
Di-n-buty1 phthalate	Cellulose acetate plastics, lacquers	Hepatic and rental effects, reduced anogential distance in males
Butylbenzy1 phthalate	Vinyl flooring, adhesives and sealants, industrial solvents	Tesicular toxicity, teratogenic
Di (2-ethylhexy1) phthalate	PVC plastics, food packging, medical devices	Hepatocellular, affects fetal growth

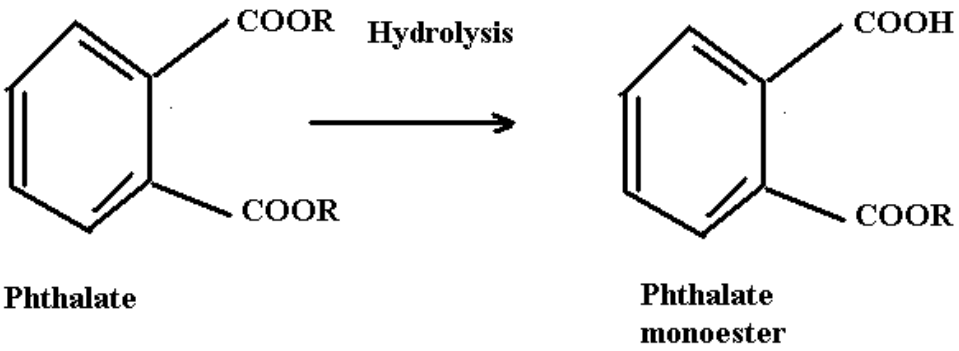

Figure 1 Generic chemical structure of phthalates diesters and phthalate - monester metabolites. R is an alkyl or aryl group

It is also shown that a clinically relevant dose and duration of exposure to DEHP has a significant effect on the behavior of cardiac cells in culture, including an uncoupling effect that results in irregular rhythms *in vitro.*

1.5.1 Metabolism

DEHP is hydrolyzed to MEHP (mono-ethyl-hexyl phthalate) and subsequently to phthalate salts (Figure 1). The released alcohol can be oxidized to the aldehyde and carboxylic acid. Generally, DEHP has a low acute toxicity. However, it is a rodent liver carcinogen and has been characterized as an endocrine disruptor. In animals, DEHP reduced testosterone production in the male fetus, potentially resulting in malformations in the external genitalia, degeneration of the seminiferous tubules, and reduced sperm production (Hauser et al., 2005; Becker et al., 2004).

2. MATERIALS AND METHODS

2.1 Physical Factors

The quality of water in a fish tank is affected by such physical factors as temperature and light. These factors crucially determine the growth and reproduction of fish. The temperature of the water in a fish tank is particularly important. A suitable rearing temperature is usually 18 to 35^{o}C. The weather in Taiwan favors the survival of zebra fish, whose spawning temperature is around 28 to 32^{o}C.

2.2 DEHP Concentration

The incubators used in this study have a length, width and height of around 90, 45 and 30 cm, respectively (121.5 liters). In the incubators, 2ml of DEHP was added to a water tank that contained 110 liters of water; 0.1 ml of DEHP was added monthly. The DEHP was analyzed by Zeyi GC chromatography. The DEHP was 99.8 % of purity. (Shelton et al.,1984; Yuan et al., 2002). Most studies of phthalates have involved a high dose and short exposure periods. However, acute exposure did not approximate real-life situations as human populations were exposed to low doses for a prolonged period. A low DEHP dosage was adopted in this experiment.

2.3 Selecting Zebra Fish

The developmental biology of zebra fish was investigated using a new method. Zebra fish are easily reared and their embryos are transparent. Therefore, in recent years, the zebra fish has become an important vertebrate animal model for research on the development of genes and human genetic diseases (Torre et al. 2002; Isabel et al. 2007).

2.4 Method

Zebra fish offspring were analyzed for three months following rapid freezing in dry ice. The zebra fish was then sent to Minxing Biotechnology Company for analysis. Mingxin Biotechnology Company examined the gene sequences of the control group and the treatment group.

2.5 DNA Analysis

Mingxin Biotechnology Company used the software ABI SOLiDTM Analyzer v4.0 for SOLiD Transcriptome Sequencing (Poly A + WT). The workflow was as follows: Sample (tissue)→ Extraction→ Isolation of poly(A) RNA (Poly(A)Purist™ MAG Kit , Ambion→ Poly(A) RNA→ Fragmentation (RNase III digestion)→ Clean-up(RiboMinusTM Concentration Module, Invitrogen)→ Fragmented RNA hybridization and adaptor ligation→ Adaptor-ligated RNA→ Reverse transcription→ Synthesized cDNA→ Purify (MinElute® PCR Purification Kit, Qiagen)→ Size selection (Novex® 6 % TBE-Urea Gel)→ Excise gels at ~150-250 bp→ Amplification→ Purification (PureLinkTM PCR Micro Kit, Invitrogen)→ Whole Transcriptome Library.

3. RESULTS AND DISCUSSION

3.1 RNA 6000 Nano Chip

The RNA 6000 Nano Chip was used for the sensitive identification of biological zene. RNA analysis was performed by an RNA 6000 Nano Chip Package. According to Figures 2- 4, the electrophoresis performance of zene was mixed. The control group contained 645.75 ng of RNA. The treatment group contained 547.14 ng of RNA.

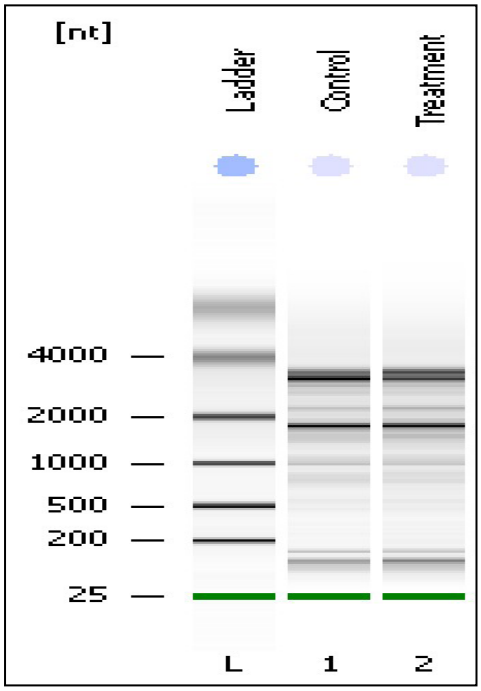

Figure 2 Electropherogram

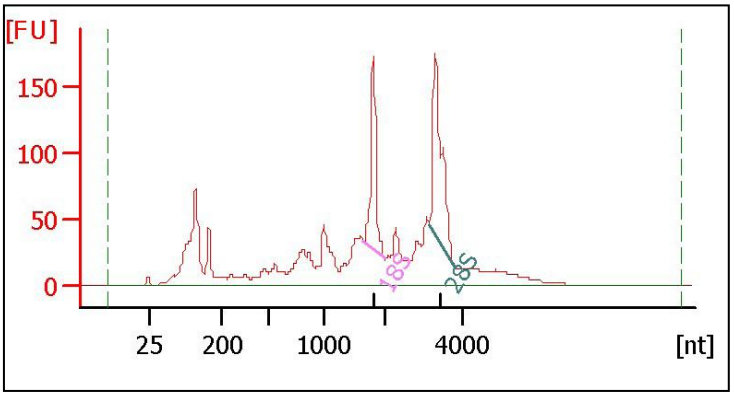

Figure 3 Electrophoresis of control group

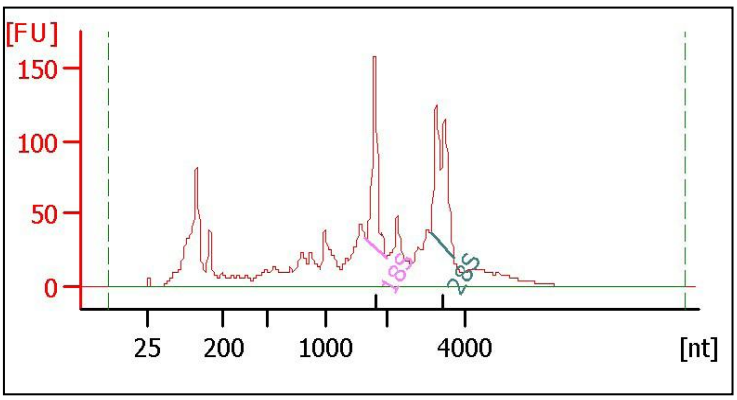

Figure 4 Electrophoresis of treatment group

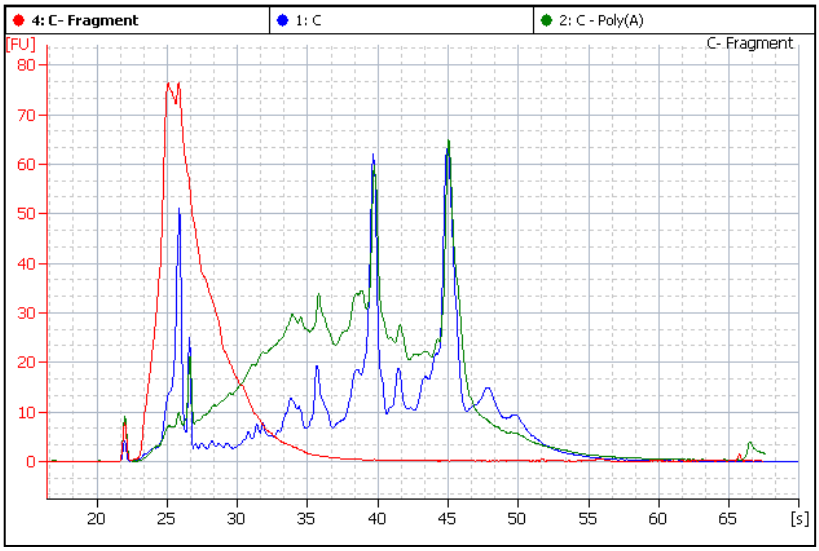

Figure 5 Gene performance of control group

Note: :Total RNA :Poly(A) RNA : Fragmented RNA

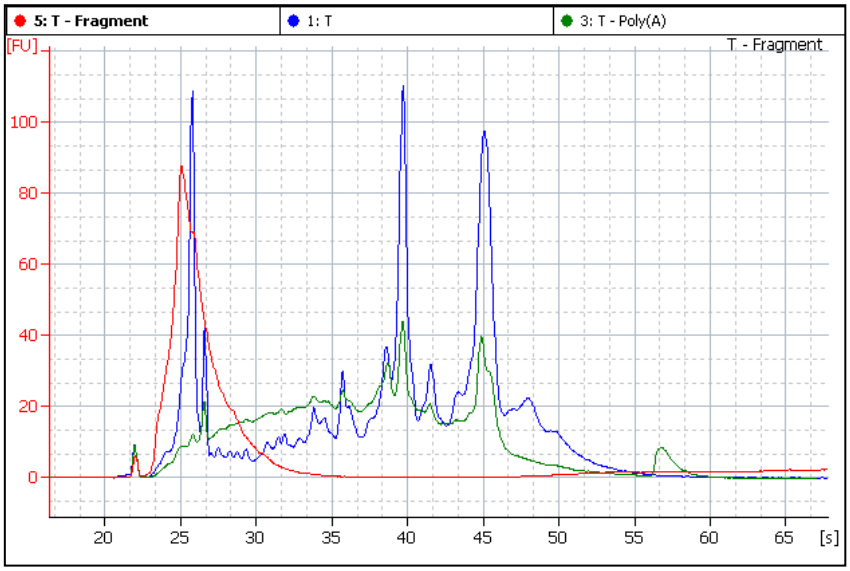

Figure 6 Gene performance of treatment group

Note: :Total RNA :Poly(A) RNA : Fragmented RNA

3.2 NanoDrop ND-1000

The NanoDrop ND-1000 was used to measure the performance in electrophoresis process of RNA. The total RNA control group contained 456.13 ng of RNA; the treatment group contained 390.06 ng of RNA. The results revealed that DEHP affected the performance of RNA. The RNA performance of the treatment group was poorer than that of the control group (Figures 5 and 6). The results of the tests that were performed using the RNA 6000 Nano Chip (Table 2) and the NanoDrop-1000 (Table 3) were consistent, and therefore reliable.

3.3 QC Test of Poly (A) and Fragmented RNA

Gel electrophoresis was utilized to compare the RNA of the blank control group with that of the treatment group. The results revealed that DEHP affected RNA.

3.4 QC Test of Whole Transcriptome Library

The fragmented DNA chosen as contaminated fragments were 150-250 bp. An existing database of zebra fish DNA gene fragments was used to compare with the gene sequence of the testing sample. The Gel-like image color of DNA performance was shown in Figures 7 and 8. DNA performance of the treatment group and the control group was varied with color. The electropherogram was used for DNA analysis, which revealed that the control group contained 336.3 ng of DNA, while the treatment group had 152.7 ng of DNA. Gene sequence analysis was conducted for the treatment group and the control group. The gene sequence of the treatment group was compared with the data in the zebra fish database.

The gene sequence results indicated that (Figures 9 and 10) the control group and the treatment group had slightly different gene sequences. DNA analysis of 50-group zebra fish genes revealed differences in gene sequences (Figure 11) in the ranges of 17-28, 35, 44-45 and 47-49. DEHP had some effects on zebra fish.

Table 2 RNA 6000 Nano Chip analysis results

No.	Sample	Vol.(μL)	Conc.(ng/μL)	aRatio	RINb	Total RNA(ng)
1	Control	250	2583	1.4	6.8	645.75
2	Treatment	250	2487	1.4	5.3	574.14

Table 3 NanoDrop ND-1000 analysis result

No.	Sample	Vol.(μL)	Conc.(ng/μL)	A260/280	A260/230	Total RNA(ng)
1	Control	250	1824.5	2.04	2.10	456.13
2	Treatment	250	1773.0	2.04	2.06	390.06

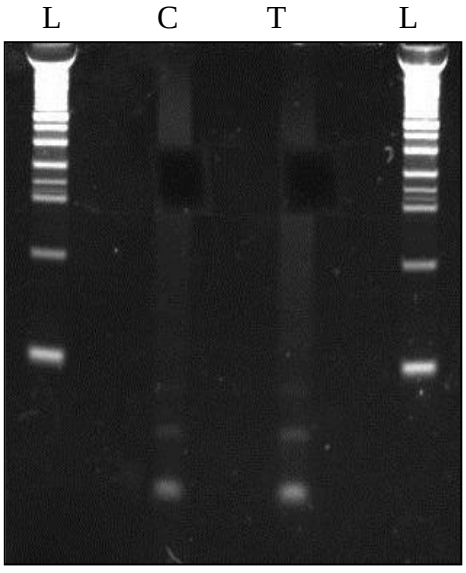

Figure 7 150-250 bp gel fragment analysis

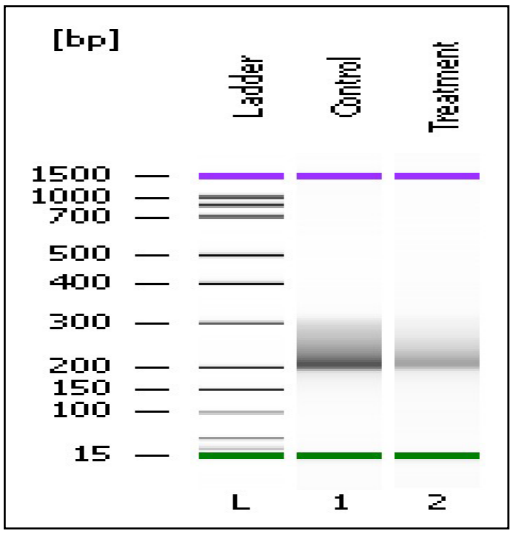

Figure 8 Electrophoresis analysis

Note: C = Control T = Treatment L = DNA Ladder,Vertical cut the gel at 150-250 bp to 4 pieces,Transfer 2 middle pieces to run PCR (2 pieces of ¼ gel ◊ 2 PCR tubes)

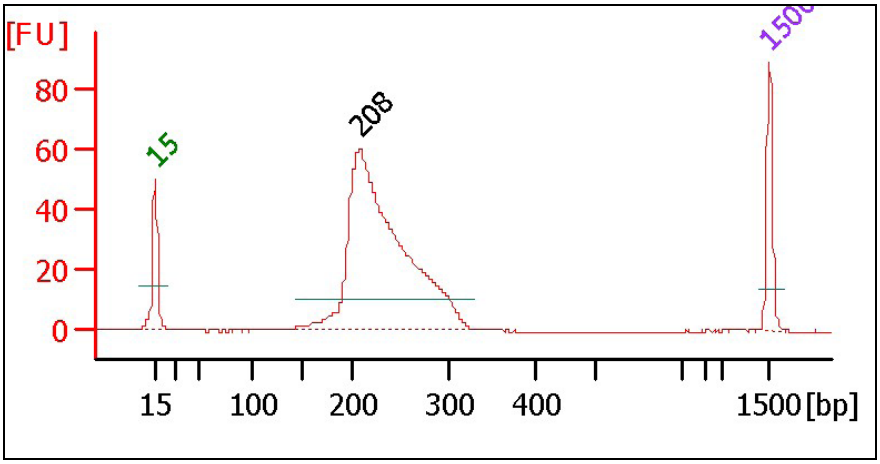

Figure 9 Gel fragment electrophoresis of control group

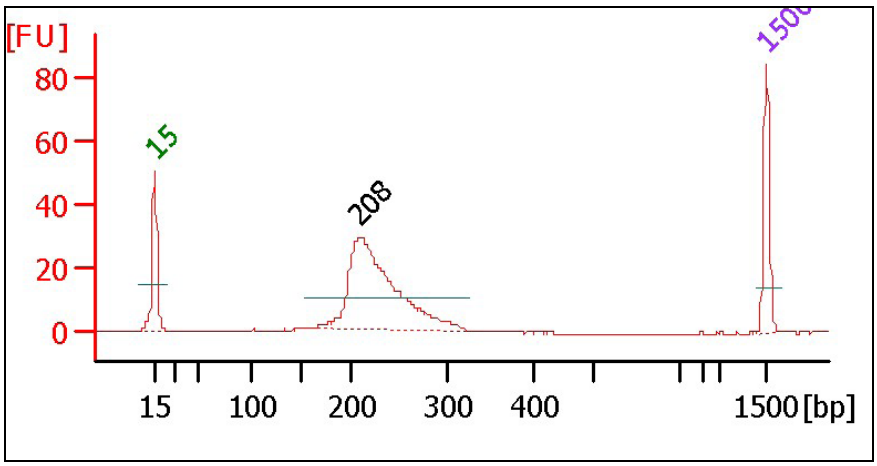

Figure 10 Gel fragment eletrophoresis of treatment group

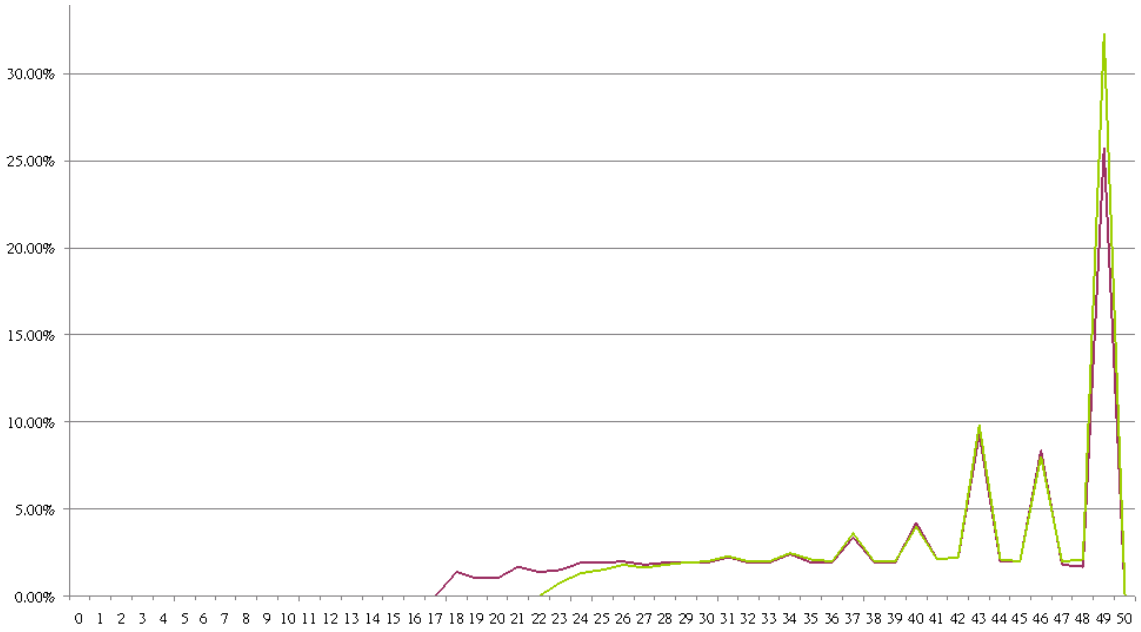

Figure 11 Corrected proportion of overlapped sequences

Note: All_Read_Aligmens (N=2180605); Primary_Read_Aligmens (N=21714082); Unigue_Read_Aligmens (N=13174234)

3.5 Analysis of Gender and Growth of Zebra Fish

The property of zebra fish in the control group was more uniformly distributed than that in the treatment group. This result was consistent with the classification of DEHP as a hormone material. The ratio of males to females in the control group was approximately (1:1), while the ratio in the treatment group was around 3:7. The body weight and length of the males in the control group were 0.474 g and 3.21 cm, respectively; the corresponding values for the females were 0.754 g and 3.10 cm. For the males in the treatment group, the values were 0.430 g and 3.01 cm, and for the females, they were 0.632 g and 3.22 cm, respectively. According to the data, DEHP influenced the growth of zebra fish.

CONCLUSIONS

In this study, the gene sequences of two groups of zebra fish were analyzed. The differences between the gene sequences of the bred generation of zebra fish in the control group and treatment group were analyzed. The variability of zebra fish genes was very slight, and occurred in zebra fish with numbers 17-28, 44, 45, 47 and 49. The gene performance of the second generation may focus on those numbers. The control group and treatment group of zebra fish, did not differ significantly in weight or length. DEHP significantly affected the gender of zebra fish in the experimental groups. Experiments revealed that the derivatives of DEHP may not be suitable for long-term consumption, and may cause unpredictable diseases.

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