1   **The acute toxicity of Oxydemeton-methyl in zebrafish**

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11   **Running title**: Toxicity of Oxydemeton-methyl in zebrafish

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14   **Key words**: Oxydemeton-methyl; toxicity; cytochrome P450; ROS; DNA damage;

15   oxidative stress; zebrafish

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24   **Abstract**

25   Oxydemeton-methyl, is an organothiophosphate insecticide, which is widely used in

26   agricultural and urban pest controls. It exists in the environment and a large amount

27   bioaccumulation in the wildlife due to its strong water solubility and mobility. Although

28   its potentially harmful effect on animals and humans, few studies have focused on the

29   oxydemeton-methyl pollution in the environment. Zebrafish have been used for many

30   years to valuate the pollution status of water and toxicity of chemicals. In the present

31   study, we aimed to investigate the effect of oxydemeton-methyl on the expression level

32   of liver microsomal cytochrome P450, on the activity of NADPH-P450 reductase and

33   reactive oxygen species (ROS) generation in zebrafish. Adult male and female zebrafish

34   were treated with different concentration of oxydemeton-methyl (10, 50, 100 μM) for 5,

35   10, 20 and 30 days. We found that the oxydemeton-methyl exposure significantly

36   increased the P450 levels and the activity of NAPDH-P450 reductase. ROS generation

37   and the DNA damage were augmented in a dose-dependent manner in the zebrafish.

38   These results indicated that oxydemeton-methyl is able to induce strong oxidative stress

39   and hence highly toxic to the zebrafish.

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47   **Introduction**

48   Oxydemeton-methyl is a widely organothiophosphate insecticide, which has

49   primarily used to control pest [1-4]. Many countries have realized the high toxicity of

50   oxydemeton-methyl to wildlife and made restriction or ban of the usage of this pesticide

51   [5-7]. However, oxydemeton-methyl remains to be one of the most frequently used

52   organophosphorus insecticides. The high water solubility and mobility led to significant

53   harmful residues in the environment.

54   Studies on toxicity testing are usually conducted in mammalian models, such as

55   rodents and rabbits [8, 9]. Whatever these tests are commonly expensive and require a

56   large amount of animals. Fish, like many other animal species in the aquatic environment,

57   has been widely used to investigate the toxicology of organophosphorus insecticides [10-

58   13]. Zebrafish has been shown to be a valuable animal model to assess the effect of

59   pollutants to the aquatic ecosystems [14-18]. Zebrafish is a useful experimental model for

60   investigating vertebrate development because of its transparent embryos, low maintaining

61   cost, conservation of key genes and signaling and easily genetic modification [19-25].

62   Hence, zebrafish has become increasingly common in compounds screening and drug

63   discovery for evaluation of the toxicity mechanisms and also in drug selection and

64   optimization [26-29]. The potential impacts of oxydemeton-methyl to the fish and the

65   increased bioaccumulation effects of the toxicant in wildlife still need be further

66   explored.

67   One of the most important manifestations in fish is oxidative stress. Increased ROS

68   levels, antioxidant defense systems impairment and loss function of the oxidative self-

69   repair can result in potential damage to fish [30-35]. The level of cytochrome P450

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70   enzymes has been well established in fish as a monitoring indicator for evaluating of

71   environmental contamination and ecotoxicology experiments [36-40].

72   In the present study, we examined the sensitivity of various biomarkers in zebrafish

73   exposed to oxydemeton-methyl, thus gain a further understanding of the impacts of

74   oxydemeton-methyl to the aquatic ecosystem.

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93   **Materials and methods**

94   **Chemicals**

95   All chemicals (analytical standard) used in this study were purchased from Sigma-

96   aldrich.

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98   **Zebrafish husbandry**

99   The wild type zebrafish (*Danio rerio*) were maintained at 28.5 °C on a 10-hours dark

100 and 14-hours light cycle. All procedures were approved from the Qingdao Municipal

101 Hospital Institutional Animal Care and Use Committee (2017N000105).

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103 **Oxydemeton-methyl exposure**

104   Six-month old adult male and female zebrafish were separated and housed in fish

105 tanks. The body weight of male and female zebrafish were 0.45 ± 0.05 g and 0.52 ± 0.08

106 g, respectively; and the length of male and female zebrafish were similar to 3.5 ± 0.3 cm.

107 Both groups were treated with or without oxydemeton-methyl (10, 50, 100 µM) and

108 samples were collected at 5, 10, 20 and 30 days post exposure. The fish were fed twice

109 daily with commercial fish food and starved overnight prior to examination to avoid the

110 effects of feces during the procedures of the assays. Half of the fish water was changed

111 daily during the period of exposure to maintain the stable concentrations of oxydemeton-

112 methyl.

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114 **Protein measurement**

115   The protein concentration was measured by using the Piece BCA Protein Assay Kit

116   (Thermo Fisher Scientific) according to the manufactures' instruction.

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118   **Isolation of liver microsomes**

119   The zebrafish were anesthetized in 0.4% tricaine (MS-222) and transferred to a moist

120   sponge for surgery on ice. The livers were dissected and rinsed with ice-cold $1\times$ PBS

121   (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, PH 7.4). The livers

122   were transferred to ice-cold homogenization buffer (0.1 M sodium phosphate buffer, 1

123   mM EDTA, 0.1 mM DTT and 0.1 mM PMSF, PH 7.5) with 10% (v/v) glycerol and

124   homogenized. The homogenate was centrifuged at 16,000 g for 20 min at 4 °C and the

125   supernatant was centrifuged at 100,000 g for 1 hour at 4 °C. The microsomal pellet was

126   collected in homogenization buffer with 20 % (v/v) glycerol and stored at -80 °C.

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128   **P450 enzyme activities**

129   The cytochrome P450 content and NADPH-P450 reductase activity were determined

130   as described somewhere else [41, 42].

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132   **Antioxidant enzymes activities**

133   The homogenate was centrifuged at 16,000 g for 20 min at 4 °C, and the supernatant

134   was collected and used for determination of the enzyme activity and protein

135   concentrations.

136   By determining the inhibition of SOD to the photochemical reduction of nitroblue

137   tetrazolium chloride (NBT), the SOD and CAT activity was measured as described

138   previously [43-46].

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140   **ROS production**

141   The ROS production was determined according to the method described previously

142   [47-49].

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144   **Statistical analysis**

145   Statistical analysis was performed using SPSS (IBM, Armonk, NY). Graphs were

146   plotted using Prism Graph Pad software (6.0). Two-way ANOVA for repeated

147   measurements used to determine the differences between duration and concentrations. All

148   the values were presented as mean $\pm$ SEM. $P \leq 0.05$ was considered statistically

149   significant by Student's t tests.

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159　**Results**

160　**Exposure to oxydemeton-methyl impaired cytochrome P450 and NADPH-P450**

161　**reductase activities**

162　　　　Compared to male fish treated with oxydemeton-methyl, the cytochrome P450

163　contents were higher in the female fish at the same exposed concentrations (Fig. 1). The

164　P450 contents were increased and reached a maximum at the 10 days and then the

165　induction showed a decreasing trend (Fig. 1). The P450 contents were higher at all

166　experiment duration in the exposed fish compared to the controls.

167　　　　The NAPDH-P450 reductase (NCR) activity of oxydemeton-methyl treated fish was

168　higher than the controls at all concentration during the exposure (Fig. 2). Oxydemeton-

169　methyl significantly induced the NCR activity, and which was higher in the female than

170　the male fish treated with the same concentrations. The NCR activity reached a maximum

171　at 20 days both in the male and female fish exposed to oxydemeton-methyl (Fig. 2).

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173　**Exposure to oxydemeton-methyl inhibited anti-oxidative enzymes activity**

174　　　　During exposure, oxydemeton-methyl significantly inhibited the SOD activity in

175　both male and female fish consistently over time (Fig. 3). The SOD activity of the female

176　fish was higher then the same concentration treated male fish.

177　　　　The CAT activity was induced by low concentration of oxydemeton-methyl (10 μM)

178　and inhibited by higher concentration and showed a consistent decrease during the

179　exposure (Fig. 4). The CAT activity in the female fish was higher than in the male fish

180　when treated with the same concentrations of oxydemeton-methyl, and which reached a

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181   maximum at 10 days. The CAT activity in the male fish reached the maximum at 20

182   days.

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184   **Effect of oxydemeton-methyl treatment on ROS production**

185   The ROS production was significantly activated by oxydemeton-methyl treatment.

186   Compared to the controls, the ROS levels in the exposed fish were higher, and had a

187   tendency to increase at all oxydemeton-methyl concentration (Fig. 5). The ROS levels

188   were higher in the female than the male fish, which may reflected some sex differences.

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Discussion

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205   Our data showed that oxydemeton-methyl exposure could affect the cytochrome

206   P450 and ROS contents, SOD NCR and CAT activities in the zebrafish. Cytochrome

207   P450 enzymes activities are useful marks that can be used in environmental

208   contamination biomonitoring and xenobiotic metabolism tests [50-52]. The P450 contents

209   were impacted by oxydemeton-methyl treatment and the stimulation trend appeared an

210   initial increase followed by a significant decrease over time. The alteration of NCR

211   would affect the function of the monooxygenase system [53]. We found that cytochrome

212   P450 affect the activity of NCR, which might be the role of P450 enzyme activity in

213   detoxification.

214   The activity of SOD and CAT was significantly induced by oxydemeton-methyl

215   treatment, which might due to oxydemeton-methyl increased ROX production in the

216   exposed zebrafish. Increased CAT and SOD activities eliminate the redundant ROS and

217   maintain ROS levels at a steady-state concentration [54, 55]. ROS, including a large

218   amount of reactive chemically molecules derived from oxygen, are generated during

219   normal physiological process in all aerobic organisms [56, 57]. ROS can directly damage

220   cellular components and affect cell function [58-60]. Oxydemeton-methyl treatment

221   significantly increased ROS levels in zebrafish, suggested its high toxicity which caused

222   the organisms were not able to eliminate the exceed ROS. The generation of ROS might

223   damage membrane lipids, DNA, protein metabolism and barbohydrate activities.

224   Our study showed that the cytochrome P450 and ROS content, SOD, CAT and NCR

225   activities were higher in female than in male fish, that suggested the sex differences can

226   affect enzyme activity. These results indicated that female zebrafish could be a good

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227   biological indicator in pollution evaluation. Our study could provide a basic theory to

228   further studies of toxicity mechanisms of oxydemeton-methyl in animals.

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451   **Figure 1 Effect of oxydemeton-methyl on the cytochrome P450 contents in zebrafish.**

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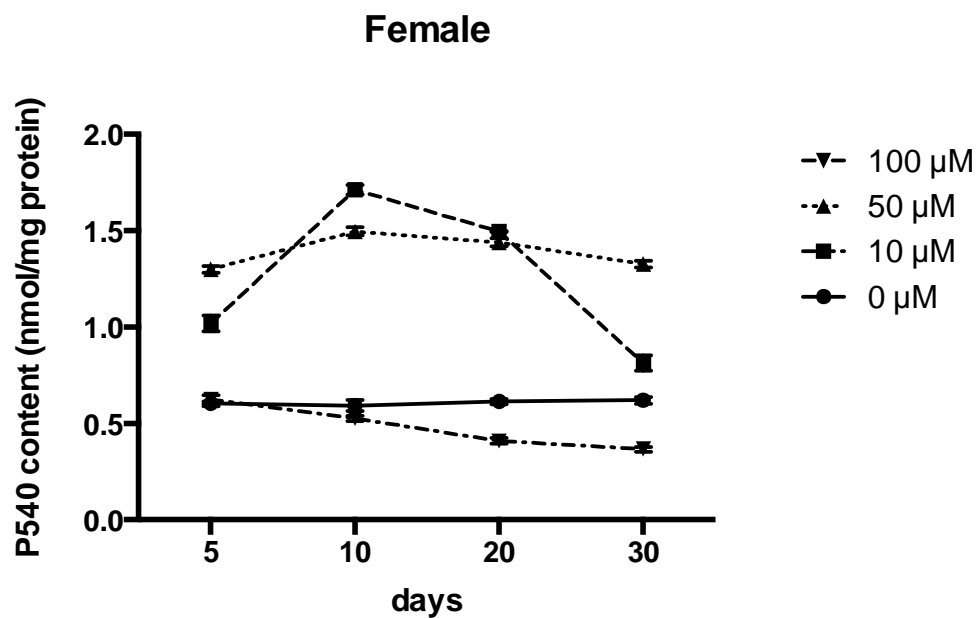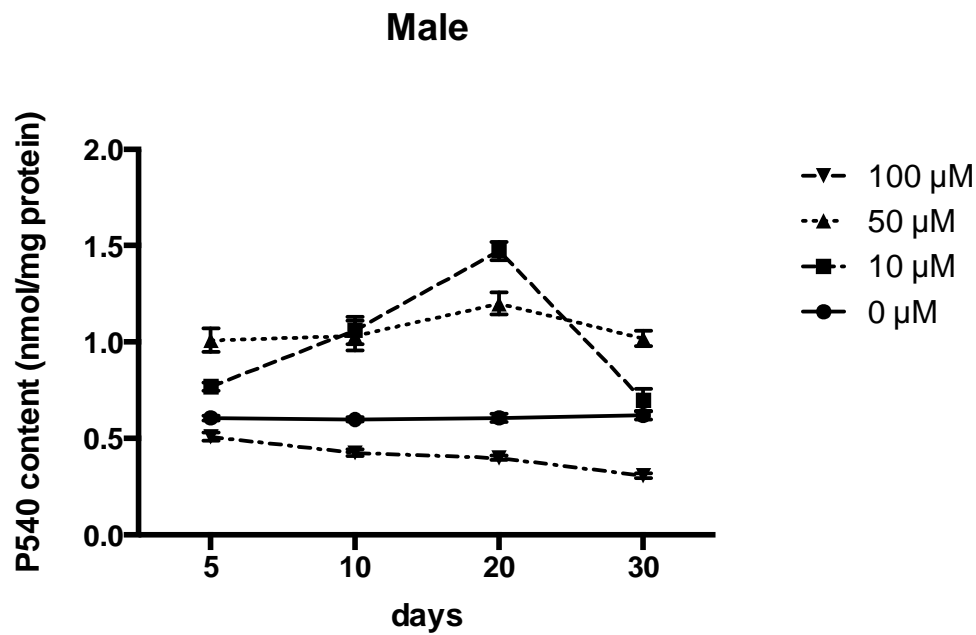
457 **Figure 2 Effect of oxydemeton-methyl on the NADPH-P450 reductase activity in**

458 **zebrafish.**

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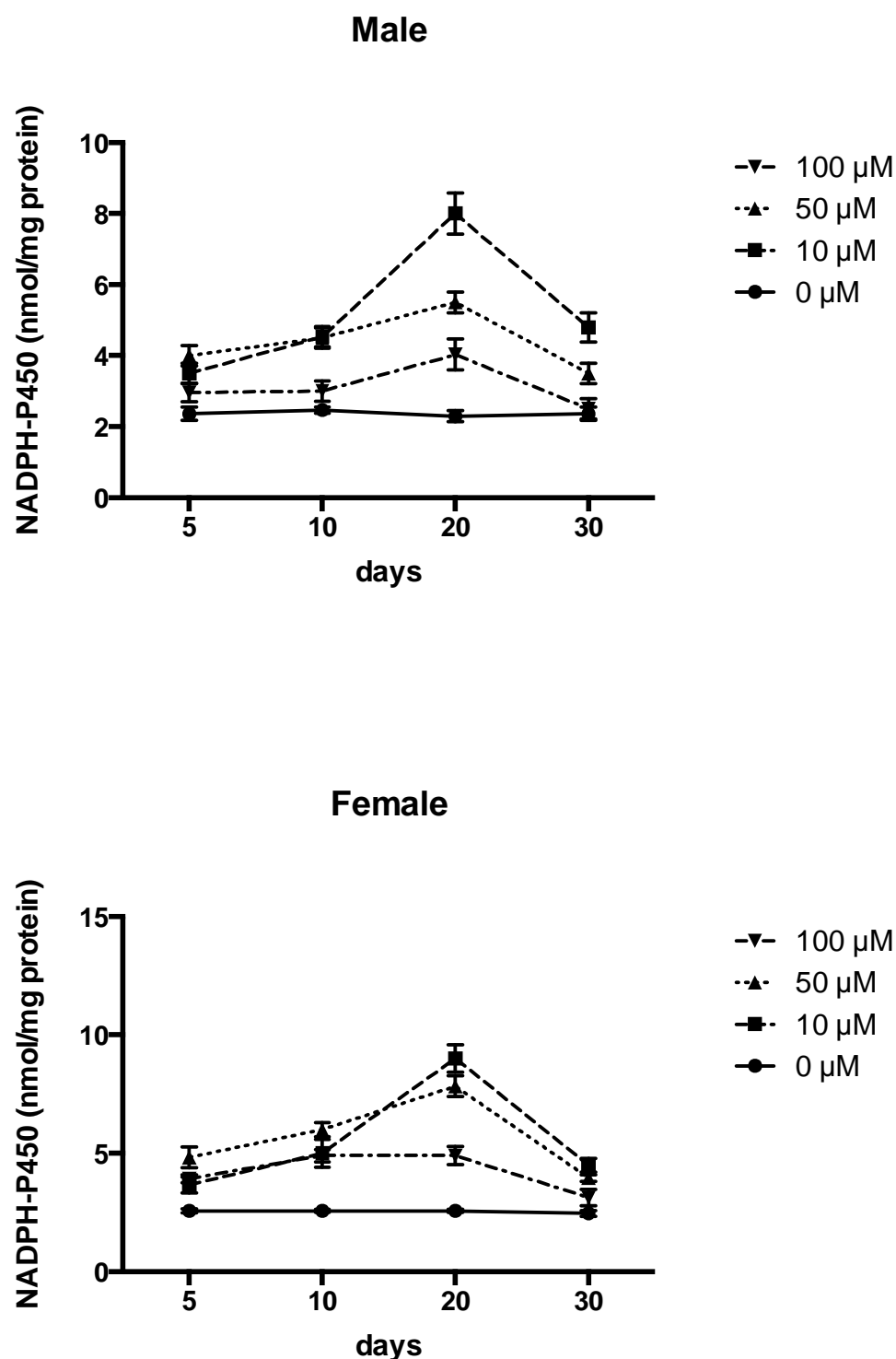
464   **Figure 3 Effect of oxydemeton-methyl on the SOD activity in zebrafish.**

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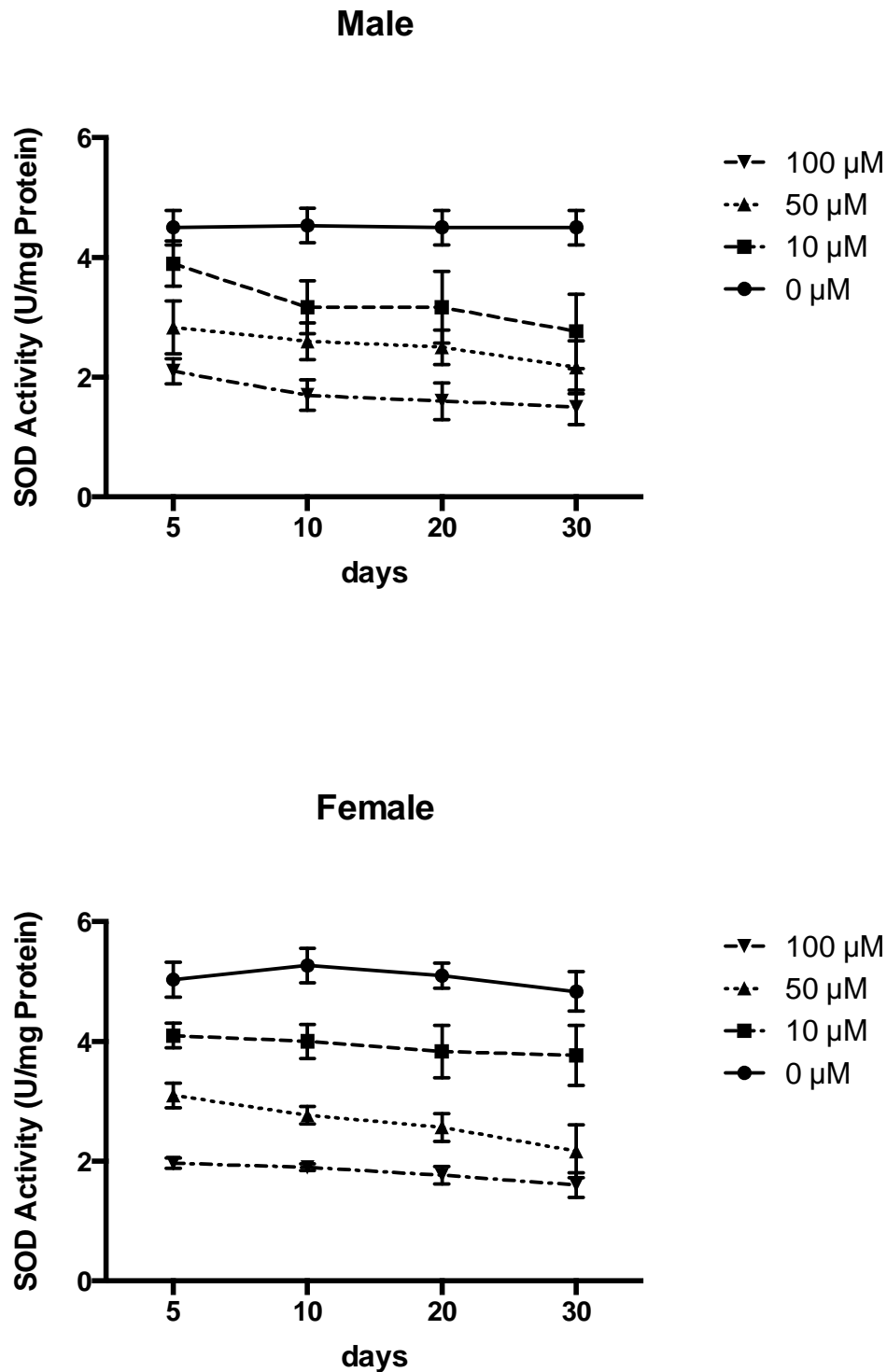
Figure 4 Effect of oxydemeton-methyl on the CAT activity in zebrafish.
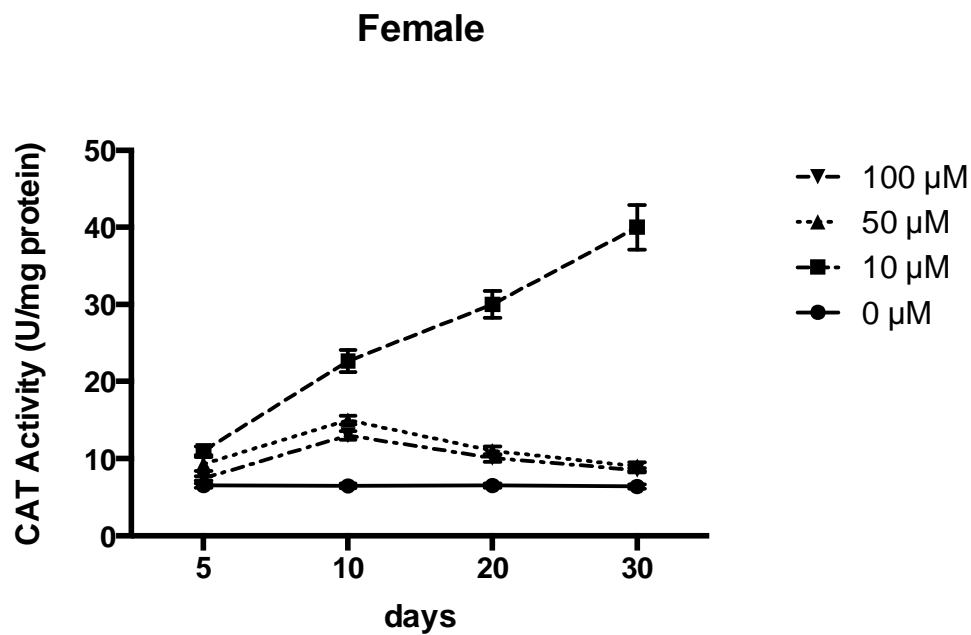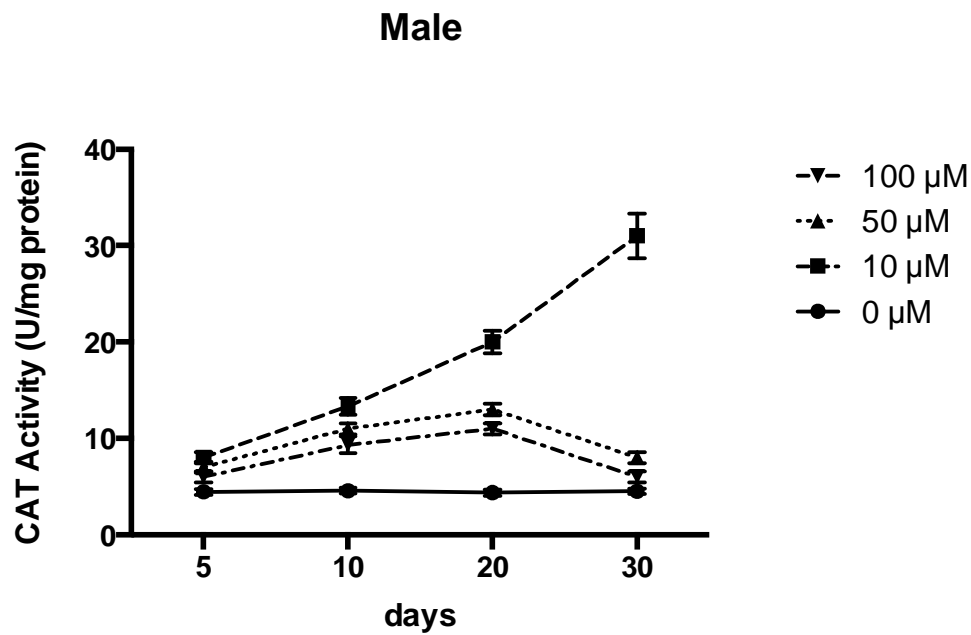
Male

Female

478   **Figure 5 Effect of oxydemeton-methyl on the ROS production in zebrafish.**

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Male

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Female

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