Effects of fragrance compounds on growth of the silkworm *Bombyx mori*

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Due to the contamination and biological toxicity of some fragrance compounds, the environmental and ecological problems of such compounds have attracted more and more attention. However, studies of the toxicity of fragrance compounds for insects have been limited. The toxicity of 48 fragrance compounds for the silkworm *Bombyx mori* were investigated in this study. All of the fragrance compounds examined had no acute toxicity for *B. mori* larvae, but eight of them (menthol, maltol, musk xylene, musk tibeten, dibutyl sulfide, nerolidol, ethyl vanillin, and α-amylcinnamaldehyde) exhibited chronic and lethal toxicity with IC_{50} values from 20 to 120 μM. In a long-term feeding study, musk tibeten, nerolidol, and musk xylene showed significant growth regulatory activity. They were also extremely harmful to the cocooning of *B. mori*, resulting in small, thin, and loose cocoons. Two important insect hormones, namely, juvenile hormone (JH) and 20-hydroxyecdysone (20-E), were quantified in hemolymph following chronic exposure to musk tibeten, nerolidol, and musk xylene, respectively. Musk tibeten significantly increased JH titer and decreased the 20-E titer in hemolymph, and musk xylene had a significant inhibitory effect on JH titer and increased 20-E titer. Although nerolidol had no effect on hormone levels, exogenous JH mimic nerolidol increased the physiological effects of JH and significantly slowed the growth rate of *B. mori* larvae. The results showed that these fragrance compounds could interfere with the insect endocrine system, leading to death and abnormal growth. The risk to insects of residual fragrance compounds in the environment is worthy of attention.

Effects of Fragrance Compounds on Growth of the Silkworm ***Bombyx mori***

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16   **Abstract:** Due to the contamination and biological toxicity of some fragrance compounds, the

17   environmental and ecological problems of such compounds have attracted more and more

18   attention. However, studies of the toxicity of fragrance compounds for insects have been limited.

19   The toxicity of 48 fragrance compounds for the silkworm *Bombyx mori* were investigated in this

20   study. All of the fragrance compounds examined had no acute toxicity for *B. mori* larvae, but

21   eight of them (menthol, maltol, musk xylene, musk tibeten, dibutyl sulfide, nerolidol, ethyl

22   vanillin, and α-amylcinnamaldehyde) exhibited chronic and lethal toxicity with IC_{50} values from

23   20 to 120 μM. In a long-term feeding study, musk tibeten, nerolidol, and musk xylene showed

24   significant growth regulatory activity. They were also extremely harmful to the cocooning of *B.*

25   *mori*, resulting in small, thin, and loose cocoons. Two important insect hormones, namely,

26   juvenile hormone (JH) and 20-hydroxyecdysone (20-E), were quantified in hemolymph

27   following chronic exposure to musk tibeten, nerolidol, and musk xylene, respectively. Musk

28   tibeten significantly increased JH titer and decreased the 20-E titer in hemolymph, and musk

29   xylene had a significant inhibitory effect on JH titer and increased 20-E titer. Although nerolidol

30   had no effect on hormone levels, exogenous JH mimic nerolidol increased the physiological

31   effects of JH and significantly slowed the growth rate of *B. mori* larvae. The results showed that

32   these fragrance compounds could interfere with the insect endocrine system, leading to death and

33   abnormal growth. The risk to insects of residual fragrance compounds in the environment is

34   worthy of attention.

35

36   **KEYWORDS:**   toxicity;   fragrance   compounds;   *Bombyx   mori*;   juvenile   hormone;   20-

37   hydroxyecdysone

38 Fragrance compounds are a group of structurally diverse additives used in a wide variety of

39 consumer products. In recent years, there have been increasing reports of pollution of fragrance

40 compounds in the environment and detection of them in organisms (Weinberg *et al.*, 2011;

41 Vecchiato *et al.*, 2018; Lou *et al.*, 2016). Tasselli et al (2021) showed the current technologies

42 are not enough efficient in removing the polycyclic musk fragrances from wastewaters in Italy.

43 The preliminary ecological risk assessment showed that synthetic musk fragrances also pose high

44 risk to aquatic organisms in the riverine and estuarine environment in Thailand (Juksu *et al.*,

45 2020). HHCB-lactone was also found with the highest concentration up to 79501 ng/g (dw) in

46 the sludge. Low removal efficiency range from -37% (HHCB-lactone) to 58% (AHTN) were

47 found for four musks. In response to the contamination and biological toxicity of some fragrance

48 compounds (such as synthetic musk fragrances), a number of studies have focused on

49 environmental and ecological problems related to them. Synthetic musk fragrances have been

50 detected in mussels (Parolini *et al.*, 2015), fishes (Blahova *et al.*, 2018), birds (Kannan *et al.*,

51 2005), marine mammals, and sharks (Nakata *et al.*, 2007; Nakata 2005), as well as in human

52 tissues and milk (Schiavone *et al.*, 2010; Moon *et al.*, 2012; Kang *et al.*, 2010). Polycyclic musks

53 acute toxicity to aquatic organisms ranges from hundreds of $\mu g\ L^{-1}$ to amounts of $< 20\ mg\ L^{-1}$

54 (Tumová et al., 2019). Schreurs *et al.* (2004) reported that AHTN and HHCB also have

55 antiestrogenic effects on the human estrogen receptor *in vitro*. Multiple studies have shown that

56 synthetic musk can increase cancer risk (Zhang *et al.*, 2017). Musk xylene exhibits a genotoxic

57 activity even after short exposure times following a DNA damage assay (Rocco *et al.*, 2015).

58 Over 80% of all of the species alive on Earth are insects. They have important significance

59   for the stable maintenance of the balance of the ecosystem (Potts *et al*., 2016). The silkworm is

60   the main raw material source of silk and thus plays an important role in human economic life and

61   cultural history. The silkworm is also widely used in entomological research as a model

62   organism. Some fragrances and flavors, especially certain essential plant oils have long been

63   reputed to repel pests (Isman, 2000). Despite the importance of silkworm and other insects, there

64   have been few studies of the effects of daily chemical fragrance compounds on insects. In this

65   study, the acute and chronic toxicity of 46 commonly used commercial fragrance compounds

66   (including natural and synthetic compounds) were performed tested to determine their lethal

67   toxicity and regulatory activity on the growth of *Bombyx mori*. The effects of the fragrance

68   compounds on cocooning were also determined in a long-term feeding study. Finally, juvenile

69   hormone (JH) and 20-hydroxyecdysone (20-E) were quantified in silkworm hemolymph to test

70   the possible causes of toxicity.

71   **MATERIALS AND METHODS**

72   ***Insect***

73   Larvae of the silkworm *Bombyx mori* strain *p50* (DaZao) were raised from eggs provided by

74   the Institute of Sericulture and System Biology at the Southwest University of China. The larvae

75   were reared on the fresh mulberry leaves at 26±1°C, 70±5% humidity with a photoperiod of 12 h

76   light and 12 h dark.

77   ***Chemicals***

78   All fragrance compounds (Table S1) were purchased from TCI Shanghai, China. JH I, JH II,

79   JH III and 20-E were purchased from Toronto Research Chemicals (Toronto, ON, Canada) and

80   Sigma-Aldrich (St. Louis, MO), respectively. The internal standards JH III-D3 and ponasterone

81   A were purchased from J&K Scientific Ltd. (Beijing, China).

Assays for to determine the impact of feeding on B. mori larval growth and mortality

83   Healthy and active larvae of similar size (larval length 3 ± 0.5 mm) at the end of the first

84   ecdysis were randomly selected and placed in plastic containers. In the acute feeding study,

85   newly molted healthy larvae at the second stadium were randomly selected and fed with a small

86   piece mulberry leaves contaminated with 2 µL fragrance solution at concentration 1 mM, and

87   then reared on normal fresh mulberry leaves. Larvae were observed after 24 h, when dead larvae

88   were recorded and removed. In the long-term feeding study, the larvae were exposed to the

89   fragrance compounds from the second instar (2 µL for insects at the second instar, 4 µL at the

90   third instar, and 6 µL at the fourth and fifth instars), administered in the same way as in the acute

91   feeding study. Each animal was treated with 2–6 pmol day^{-1} (approximately 1 ng day^{-1}) fragrance

92   compounds. Each day, after the larvae had finished ingesting the contaminated mulberry leaves,

93   fresh and clean leaves were fed to maintain their dietary requirements. The fragrance compounds

94   were dissolved in dimethyl sulfoxide and then mixed with pure water. The concentrations ranged

95   from 0.001 to 10 mM. The concentrations tested for each compound were shown in Table S1.

96   Larval mortality and insect wet weight were recorded every day after treatment (Wang *et al*.,

97   1999). Controls were treated with the solvent. There have three replications (done on separate

98   days) at each dose and each replication contained 5–7 larvae.

Quantitative assays of JH in hemolymph using LC-MS/MS

100   Newly molted fifth-instar *B. mori* (day 0) were used in this study. The hemolymph was

101   collected in a microsyringe after cutting an abdominal leg. A volume of 50 μL hemolymph was

102   immediately transferred to a 500 μL glass centrifuge tube containing 50 μL acetonitrile, 50 μL

103   sodium chloride (0.9%, wt v^{-1}) solution, and 5 ng JH III-D3 as an internal standard. The sample

104   was extracted twice with 100 μL hexane following vigorous vortexing and centrifuged at 2500 ×

105   g for 5 min (Kai *et al*., 2018). The hexane phase (upper layer) was removed and transferred to a

106   new glass vial, and then dried under nitrogen flow. The residue was dissolved in 1 mL

107   acetonitrile. The measurement of JHs was determined using a liquid chromatography method

108   coupled to electrospray tandem mass spectrometry (LC-MS/MS) reported by Ramirez et al.

109   (2020).

110   ***Quantitative assays of 20-E in whole body using UPLC-MS/MS***

111   The hemolymph of *B. mori* larvae (4 days into the fourth instar) was collected as described

112   above, and 50 μL hemolymph was immediately transferred into 500 μL methanol. Ponasterone A

113   (10 ng mL^{-1}) was used as the internal standard in this study. The mixture was vortexed

114   vigorously and centrifuged at 2500 × g for 10 min. The supernatant was collected in a new

115   microtube, and the pellet was re-extracted with 500 μL methanol. The combined methanol

116   extracts were evaporated under a stream of nitrogen. The residue was dissolved in 100 μL 80%

117   methanol in water (Furuta *et al*., 2010). The quantitative assay for 20-E was determined using a

118   UPLC-MS/MS system, consisting of Waters ACQUITY UPLC I-Class (Waters Corp., Milford,

119   MA), coupled with AB SCIEX TRIPLE QUAD™ 5500 mass spectrometer from AB Sciex

120   (Framingham, MA, USA).

121   An ACQUITY UPLC®BEH C18 column (2.1 mm × 100 mm, 1.7 μm) from Waters was

122   used for separation at 40°C with a sample injection volume of 3 μL. A binary mobile phase was

123   composed of (A) methanol and (B) 0.1% formic acid in water. A linear mobile phase gradient

124   started at 10% A (0–1 min) and went to 90% A at 4 min (held until 7 min), followed by a

125   gradient to 10% A at 7.1 min (held until 8 min) at a flow rate of 0.3 mL min^{-1}. The MS

126   determination was performed in positive electrospray mode with monitoring of the two most

127   abundant MS/MS (precursor/product) ion transitions using scheduled multiple-reaction

128   monitoring (MRM) program for each analyte. The ion source conditions were as follows: ion

129   spray voltage, 5.5 kV; block source temperature, 500°C; ion source gas 1 pressure, 50 psi; ion

130   source gas 2 pressure, 50 psi; curtain gas pressure, 38 psi; and collision gas (argon) pressure, 8

131   psi. Data acquisition and processing were carried out by Analyst software version 1.6.1 (Applied

132   Biosystems, Foster City, CA, USA). The retention times for 20-E and ponasterone A were 3.86

133   min and 4.43 min, respectively. The quantification transition for 20-E was 481.3→445.3

134   (declustering potential [DP]: 132 V, CE: 24 V). The confirmation transitions were 481.3→371.2

135   (DP: 159 V, CE: 23 V) and 481.3→427.5 (DP: 138 V, CE: 23 V), respectively. For the internal

136   standard ponasterone A, the quantification transition was 465.5→429.3 (DP: 150 V, CE: 23 V),

137   and the confirmation transitions were 465.5→295.3 (DP: 150 V, CE: 30 V) and 465.5→411.2

138   (DP: 160 V, CE: 26 V).

139   ***Statistics***

140   Comparisons of the effects of fragrance compounds on larval stadium and the growth of

141   larva were analyzed with a pooled *t*-test. The threshold for statistical significance was set at 95%

142   (P = 0.05). Data were presented as percentages and were log-transformed before statistical

143   analyses. Dose-response curves were prepared using the software GraphPad Prism version 5.0.

144   Values are expressed as means ± standard errors (SEM), with N indicating the number of

145   samples measured (N = 15–21).

146   **RESULTS**

147   ***Larval mortality following oral administration***

148   A stomach toxicity test was performed to determine the larvicidal effects of the fragrance

149   compounds in this study. After 24 h exposure following feeding at concentration 1 mM, no

150   larvae died. This suggests that the fragrance compounds in this study have no acute toxicity for *B.*

151   *mori* larvae. However, eight fragrance compounds (menthol, maltol, musk xylene, musk tibeten,

152   dibutyl sulfide, nerolidol, ethyl vanillin, and α-amylcinnamaldehyde) showed chronic toxicity.

153   Most larvae that commenced feeding with these eight compounds from the second stadium died

154   prior to pupation. The LC_{50} value and mortality at 0.1 mM of each compound are shown in Table

155   1. These results show that the eight fragrance compounds have chronic and lethal toxicity for *B.*

156   *mori* larvae. In the dead larvae, the most striking characteristic was the darkening of the cuticle

157   in some animals and molting disturbances. This phenomenon suggested that these fragrance

158   compounds might interfere with insect hormone levels, leading to death (Monger *et al.*, 1982).

159   No antifeedant effect was found in these experiments.

160   ***Growth regulation***

161   A long-term feeding study with fragrance compounds at 1 μM was performed to identify

162   their effects on growth. We fed larvae with fragrance compounds starting at the second instar

163   (Day 0), and recorded the number of days from hatching to pupation (larvae that died before

164   pupation were not recorded). Figure 1 shows musk tibeten and nerolidol significantly slowed the

165   growth rate of *B. mori* larvae. However, musk xylene caused precocious pupation (Figure 1). For

166   musk tibeten, nerolidol and musk xylene, the times from newly hatched larvae to pupation were

167   31.6, 29.9, and 24.4 days, respectively. In the control group (not fed inhibitors), the interval was

168   27.4 days. Figure 2 clearly shows that larvae fed with these three fragrance compounds were

169   significantly smaller than the control group. Four larval groups were hatched on the same day

170   (Day 0, second instar); one was fed normal food as control, and the others were treated with 1

171   μM musk tibeten, nerolidol, and musk xylene, respectively. The difference in wet weight was

172   readily apparent by comparison with the control insects at the fifth instar.

173   The effects of musk tibeten, nerolidol, and musk xylene on cocooning of *B. mori* were also

174   determined with a long-term feeding study at 1 μM as above. The mean wet weight of cocoon in

175   treatment was significantly less than in the controls (Figure 3). Compared to the control, the wet

176   weight of cocoon exposed to musk tibeten, nerolidol, and musk xylene lost 0.34 g cocoon^{-1}, 0.23

177   g cocoon^{-1}, and 0.40 g cocoon^{-1}, respectively. There were no significant differences between

178   treatments and the controls on rate of cocooning.

179   Although mortality in the low-dose chronic exposure to fragrance compounds was minimal,

180   the growth of *B. mori* larvae was greatly interfered with, and the mean wet weight of larvae in

181   each larval instar were also reduced following chronic exposure to musk tibeten, nerolidol, and

182   musk xylene. These three fragrance compounds were also extremely harmful to the cocooning of

183   *B. mori*. The affected larvae exhibited behavioral responses, such as obstruction of cocoon-

184 making or spinning, feeble pulse rate and final death of surviving larvae. Some larvae made only

185 thin and loose cocoons.

186 ***Effects on JH and 20-E titer in hemolymph by treatment with fragrance compounds***

187   Hormones are generated and used by insects to regulate physiological, developmental, and

188 behavioral events. Juvenile hormones and ecdysteroids are two important categories of insect

189 hormone. Ecdysteroids are a family of steroid hormones. Their most common form in insects is

190 20-E, which is converted from ecdysone in the fat body or epidermis (Horn *et al*., 1966).

191   To study the possible causes of chronic toxicity, JHs (JH I, JH II and JH III) and 20-E were

192 quantified in hemolymph following chronic exposure to musk tibeten, nerolidol, and musk

193 xylene. Larvae were fed fragrance compounds (an approximate exposure of 1 ng day^{-1}), as

194 described in Section 3.2. Figure 4 shows that musk tibeten and musk xylene had significant

195 effects on JHs titer following treatment *in vivo*, while nerolidol was inactive. It is interesting that

196 musk tibeten significantly increased JHs titer in hemolymph, whereas musk xylene had

197 significant inhibitory effect. In this study, other synthetic musk fragrances (such as musk ketone,

198 musk AHMT, and phantolide) did not demonstrate any growth-regulatory effects on *B*. *mori*.

199   The 20-E titer in the hemolymph of *B*. *mori* larvae (day 4 of the fourth instar) was

200 determined by UPLC-MS/MS (Figure 5). Treatment with musk tibeten significantly decreased

201 the 20-E titer in hemolymph, and musk xylene had a significantly increased effect. This result

202 was contrary to the JH titer. Musk tibeten increased JH titer but reduced 20-E titer in hemolymph,

203 which resulted in the retardation of the growth of *B*. *mori* larvae. Must xylene had a significant

204 inhibitory effect on JH titer but increased the 20-E titer in hemolymph. This caused precocious

205   pupation. Nerolidol also had no effect on 20-E titer. The quantitative assays for JHs and 20-E

206   titer indicate possible causes of growth regulation by these three fragrance compounds.

207   **DISCUSSION**

208   Geraniol and geranylgeraniol have been shown to inhibit JH biosynthesis by the corpora

209   allata in lepidopteran and blattarian insects in vitro (Kai et al., 2018; Sperry and Sen, 2001).

210   Coumarin has been demonstrated as an ovicide inhibitor against fruit fly Drosophila

211   melanogaster (Shuhei and Kazuyoshi, 1980). Toxicity tests illustrated the fumigant toxicity of

212   1,8-cineole against stored-grain insects (Lee et al., 2004). However, these four fragrance

213   compounds above had no lethal toxicity to *B. mori* larvae following the stomach toxicity test in

214   this study. This suggests that different species of insects have different sensitivities to given

215   fragrance compounds.

216   Our result suggests that the structural differences in fragrances have a great influence on

217   toxicity. Musk tibeten and musk xylene are aromatic nitro compounds, and musk ketone has an

218   aldehyde group in addition to the nitro group, however musk AHMT and phantolide do not

219   contain nitro groups. Nerolidol is a mimic of JH, which has a similar function, but it has no

220   effect on JH biosynthesis and metabolism (Burdette, 1964). In hemolymph, exogenous JH mimic

221   nerolidol increases the physiological effects of JH and significantly slows the growth of *B. mori*

222   larvae.

223   In previous studies, some fragrance compounds have been demonstrated to have contact,

224   fumigant, antifeedant, attractant, and repellent insecticidal actions against specific pests (Isman,

225   2000). Due to the extensive use of fragrance compounds and the growth of the fragrance industry,

226 fragrance residues can be detected in the environment. Concentrations of fragrance compounds

227 in wastewater were found in concentration at μg L^{-1} in a study in Italy (Tasselli *et al.*, 2021). In

228 the long-term feeding study with fragrance compounds at 1 μM was performed to identify their

229 effects on the growth of insect. Residual fragrance compounds may cause damage to the

230 sericulture industry and may be harmful to other insects in the environment. The current leakage

231 concentration may not immediately affect the ecology of insects. However, there are concerns

232 about the deterioration of environmental pollution in the future.

233 **CONCLUSIONS**

234 Although the fragrance compounds in this study have no acute toxicity for *B. mori* larvae,

235 some exhibit chronic and lethal toxicity. In a long-term feeding study, musk tibeten, nerolidol,

236 and musk xylene have significant growth-regulatory activity with an approximate exposure of 1

237 ng day^{-1}. They were also extremely harmful to the cocooning of *B. mori*. Larval exposure to

238 these fragrance compounds produced small, thin, and loose cocoons. The quantitative assays for

239 JH and 20-E titer in hemolymph showed that these fragrance compounds could interfere with

240 hormone levels in insects, leading to death and abnormal growth. The risk to insects of residual

241 fragrance compounds in the environment is worthy of attention.

242

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346

347 **Figure 1.** Number of days from hatching to pupation following a long-term feeding study with

348 musk tibeten, nerolidol, and musk xylene at 1 μM. *B. mori* larvae were fed with fragrance

349 compounds starting with the second instar, and the number of days from hatching to pupation

350 was recorded. Values represent means ± SEMs, ****$p < 0.0001$.

351 **Figure 2.** Effects on larval weights of *B. mori* at the fifth instar in the chronic toxicity assay.

352 Four larval groups hatched on the same day (Day 0, second instar); one was fed normal food as

353 control, and the others were treated with 1 μM musk tibeten, nerolidol, and musk xylene,

354 respectively. Each point represents mean ± SD. $N = 20$-21 individuals.

355 **Figure 3.** Effects of musk tibeten, nerolidol, and musk xylene on the mean wet weight of cocoon

356 in the chronic toxicity assay. Values represent means ± SEM, ****$p < 0.0001$.

357 **Figure 4.** Effects of musk tibeten, nerolidol, and musk xylene on hemolymph JHs titer of newly

358 molted fifth instar *B. mori* (day 0). Values represent means ± SEM, ****$p < 0.0001$.

359 **Figure 5.** Effects of musk tibeten, nerolidol, and musk xylene on hemolymph 20-E titer of *B.*

360 *mori* larvae on the fourth day of the fourth instar. Values represent means ± SEM, ****$p <$

361 0.0001.

The LC$_{50}$ values of fragrance compounds in the chronic toxicity assay

The LC$_{50}$ values of fragrance compounds in the chronic toxicity assay

1 **Table 1.** The LC_{50} values of fragrance compounds in the chronic toxicity assay

Compound	LC_{50} value (μM) (95% CI) (nonlinear fit model, df ; R^2)	Mortality at 0.1 mM (%)
Menthol	76.6 (46.6 – 125.9) (df = 25; R^2 = 0.95)	67 ± 3
Maltol	56.3 (39.2 – 80.8) (df = 29; R^2 = 0.96)	67 ± 3
Musk xylene	60.7 (29.3 – 125.6) (df = 21; R^2 = 0.92)	67 ± 4
Musk tibeten	88.1 (52.3 – 148.4) (df = 27; R^2 = 0.97)	65 ± 5
Dibutyl sulfide	118.1 (66.2 – 210.7) (df = 27; R^2 = 0.90)	51 ± 5
Nerolidol	63.2 (23.5 – 170.0) (df = 25; R^2 = 0.85)	66 ± 15
Ethyl vanillin	23.8 (10.8 – 52.4) (df = 23; R^2 = 0.95)	100 ± 0
α-amylcinnamaldehyde	96.2 (46.5 – 199.0) (df = 25; R^2 = 0.93)	55 ± 8

2 The repeat number of each concentration was 15−21; CI = confidence intervals; df = degrees of

3 freedom.

Figure 1

Number of days from hatching to pupation following a long-term feeding study with musk tibeten, nerolidol, and musk xylene at 1 µM.

B. mori larvae were fed with fragrance compounds starting with the second instar, and the number of days from hatching to pupation was recorded. Values represent means ± SEMs, ****$p < 0.0001$.
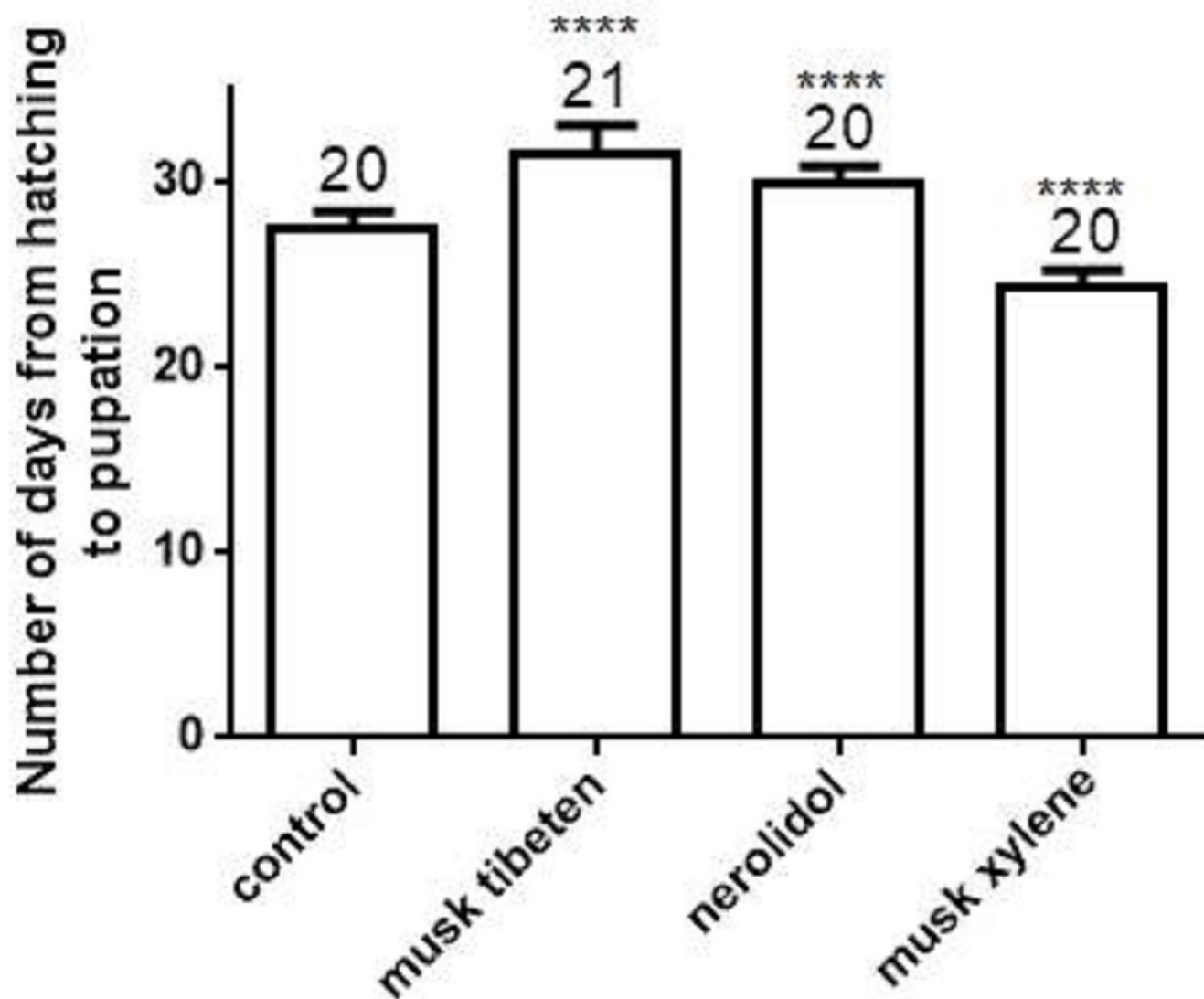
Figure 2

Effects on larval weights of *B. mori* at the fifth instar in the chronic toxicity assay.

Four larval groups hatched on the same day (Day 0, second instar); one was fed normal food as control, and the others were treated with 1 µM musk tibeten, nerolidol, and musk xylene, respectively. Each point represents mean ± SD. *N* = 20-21 individuals.
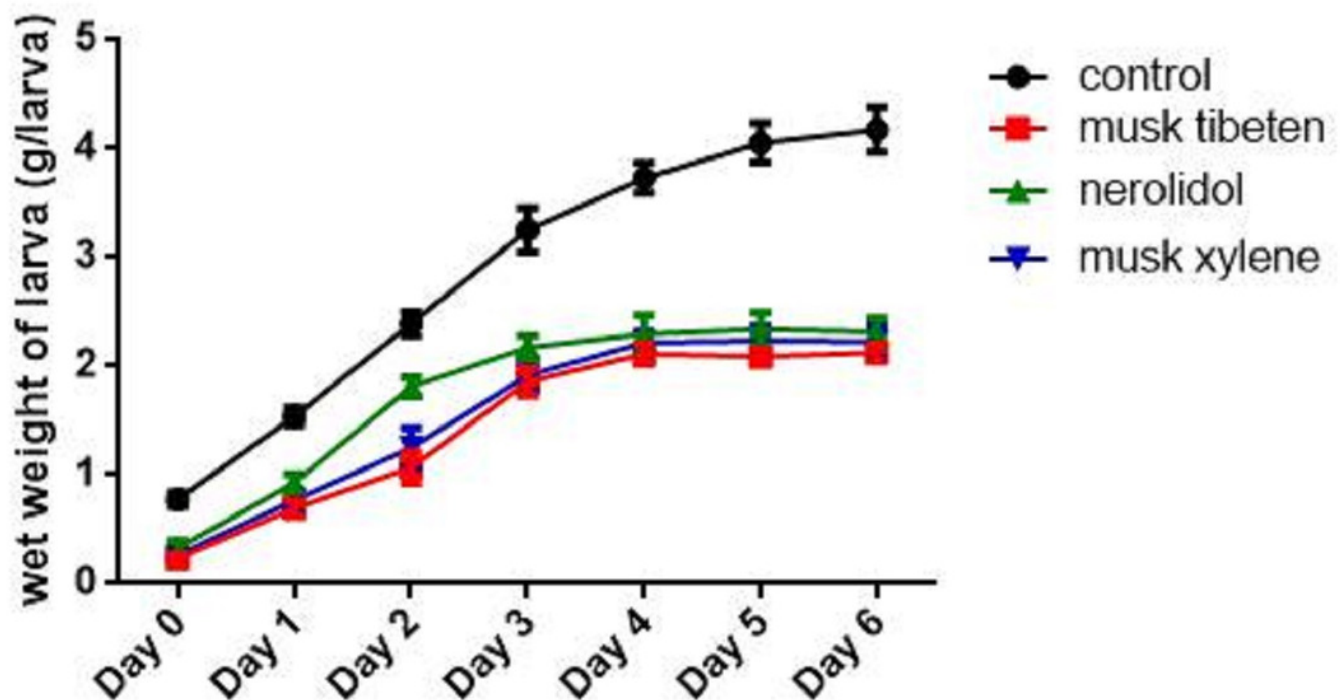
Figure 3

Effects of musk tibeten, nerolidol, and musk xylene on the mean wet weight of cocoon in the chronic toxicity assay.

Values represent means ± SEM, ****$p < 0.0001$.
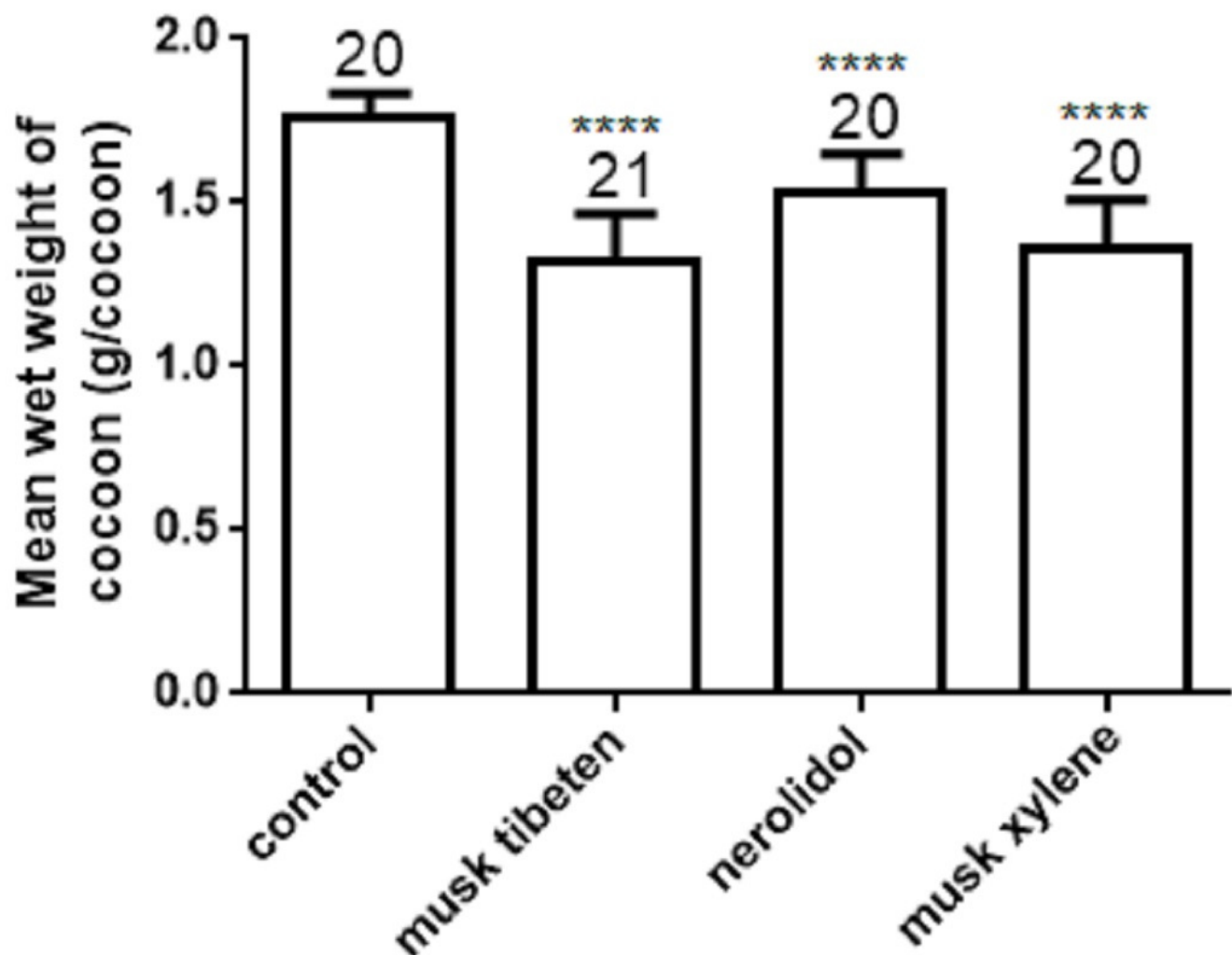
Figure 4

Effects of musk tibeten, nerolidol, and musk xylene on hemolymph JHs titer of newly molted fifth instar *B. mori* (day 0).

Values represent means ± SEM, ****$p < 0.0001$.
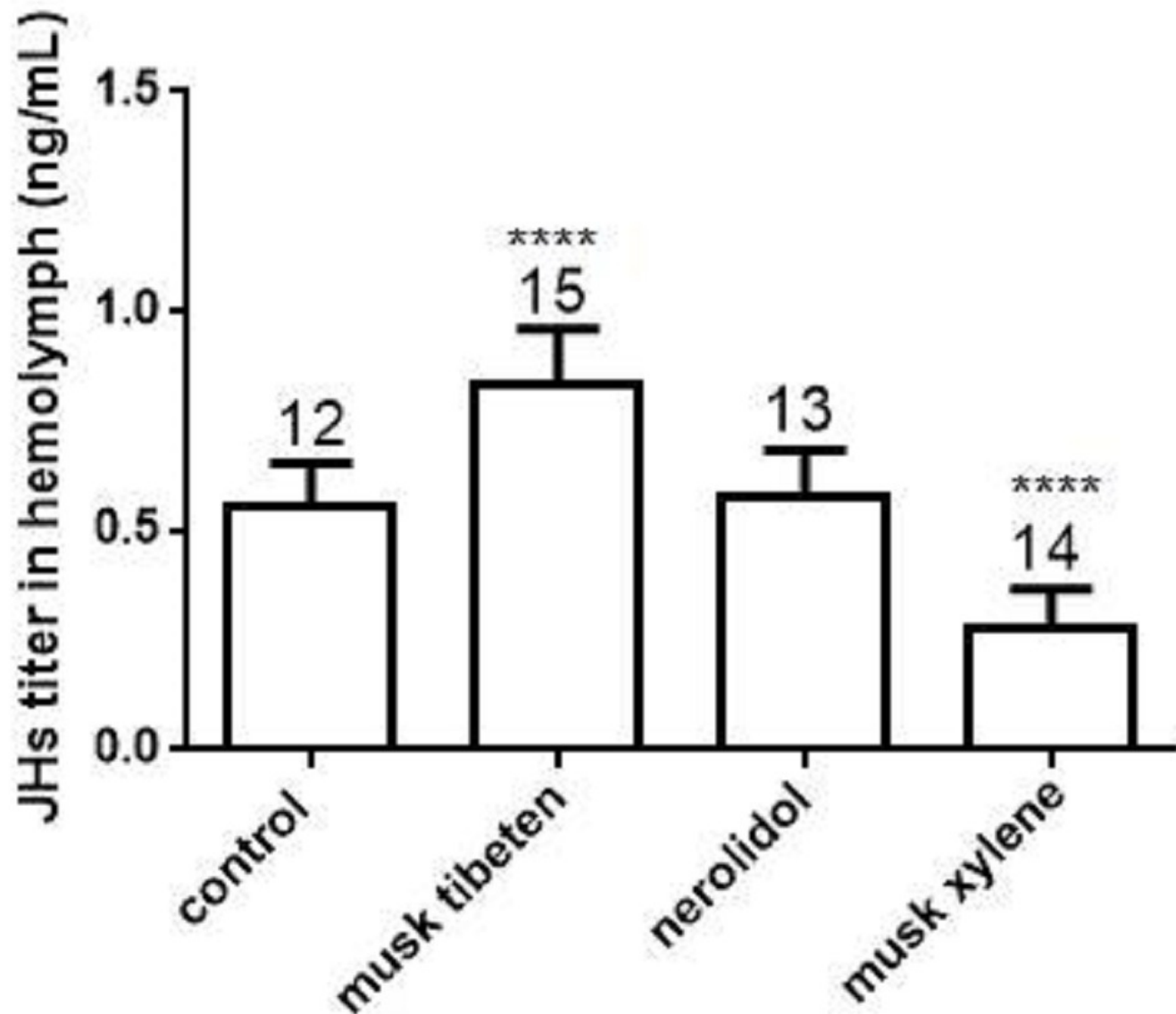
Figure 5

Effects of musk tibeten, nerolidol, and musk xylene on hemolymph 20-E titer of *B. mori* larvae on the fourth day of the fourth instar.

Values represent means ± SEM, ****$p < 0.0001$.