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2 **Estrogenic action by tris(2,6-dimethylphenyl) phosphate, an impurity in**
3 **resorcinol bis[di(2,6-dimethylphenyl) phosphate] flame retardant formulations,**
4 **impairs the development of female reproductive functions**
5
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31 The authors declare they have no actual or potential competing financial interests.
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33
34
35
36 ## Abstract
37

38 **Background**: Developmental exposure to environmental chemicals with estrogen-like
39 activity has been suspected to permanently impair women's health.
40 **Objectives**: In this study, we used a mouse model to evaluate whether a chemical
41 having putative estrogen-like action detected by *in vitro* study, namely
42 tris(2,6-dimethylphenyl) phosphate (TDMPP) impairs sexual differentiation of the
43 brain.
44 **Methods**: To induce developmental exposure, TDMPP was administered
45 subcutaneously to dams from gestational day 14 to parturition and to pups from
46 postnatal day 0 to 9 at two different doses (TDMPP-high and TDMPP-low groups,
47 respectively). To compare the results between TDMPP and typical estrogen exposures,
48 17β-estradiol was administered at two different doses on the same treatment schedule
49 (E_2-low and E_2-high groups, respectively). A vehicle control group was formed by
50 administering an equivalent volume of sesame oil to dams and to pups.
51 **Results**: Although there was no specific impairment in female ovary morphology,
52 precocious puberty, detected by vaginal opening, and irregular estrous cycles,
53 detected by vaginal cytology after sexual maturation, were found in TDMPP- and
54 E_2-treated groups, but not in the vehicle control group. In addition, lower lordosis
55 response during reproductive behavioral tests was found in TDMPP- or E_2-treated
56 groups. To further clarify whether TDMPP directly affects sexual differentiation of
57 the brain, we evaluated the transfer of TDMPP into the brain and the formation of
58 sexual dimorphic nuclei. We detected a certain amount of TDMPP and its metabolites
59 in the mouse brain after treatment, and masculinization of sexual dimorphic nuclei in
60 the hypothalamus of female mice, suggesting the direct impact of TDMPP in
61 developing brain.
62 **Discussion**: Taken together, the experimental evidence demonstrates that TDMPP
63 directly enters the fetal and neonatal brain, inducing changes of sex-related brain
64 structures, and impairing female reproductive functions.
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67 **Introduction**

68

69 One of the "Sustainable Development Goals" accepted by the United Nations in
70 2015 is gender equality (United Nations Statistical Commission, 2016). In the field of
71 environmental health, gender-specific health issues related to sustainable development
72 have gained increasing attention, and the inclusion of sex and gender differences in
73 environmental health research has been proposed (Langer et al., 2015). Among
74 environmental health problems related to sex and gender, sex difference of the
75 biological responses when exposed to chemicals should be addressed more
76 intensively, because the current male-bias in the use of experimental animals
77 (Prendergast et al., 2014), even when evaluating the toxicity of new products, might
78 prevent the detection of harmful effects in females. In particular, the effect of
79 endocrine-disrupting chemicals has been known to be considerably sex-biased, and
80 the effect of the exposition to endocrine disruptors in a vulnerable period such as the
81 perinatal period is especially serious because of the permanence of the effects
82 (Diamanti-Kandarakis et al., 2009). Therefore, the systematic evaluation of the
83 impairment of sexual differentiation by new products using both males and females is
84 required to elucidate their harmful effects.

85 In mammals, sexual differentiation of physiology and behavior during development
86 is controlled not only by sex chromosomes, but also by gonadal steroid hormones. It
87 has been reported that testosterone secreted from the testes during the developmental
88 period in rodents is critical for determining the orientation of various kinds of
89 behaviors, including sexual and social behaviors (Phoenix et al., 1959). In empirical
90 studies using rodents, neonatal injection of testosterone was reported to cause
91 abnormality in the estrous cycle, detectable by vaginal cytology, and to decrease
92 female reproductive receptivity to males after sexual maturation, detectable by a
93 typical receptive behavior called lordosis (Maekawa et al., 2014). Since the
94 impairments in female-specific physiological and behavioral parameters have also
95 occurred by neonatal injection of estrogen instead of testosterone, estrogen converted
96 from testosterone in the brain by aromatase is thought to mediate them (MacLusky
97 and Naftolin, 1981). Not only endogenous estrogen, but also chemicals with
98 estrogenic activity, known as xenoestrogens, have been known to impair the
99 development of female physiology and behavior. Estrogen affects sexual
100 differentiation of the brain by acting on estrogen receptor alpha (ER-α), because the
101 typical sexually dimorphic nuclei of the preoptic area (SDN-POA) and the bed
102 nucleus of the stria terminals (BnST) were reported to develop in an ER-α dependent
103 manner (Patchev et al., 2004; Tsukahara et al., 2011). Among xenoestrogens,
104 bisphenol-A, used in the manufacturing of polycarbonate products, has become
105 notorious, because a considerable amount of empirical evidence shows that it exerts
106 its endocrine-disrupting action at least in part through ER-α (Gould et al., 1998),
107 even if other estrogen receptors could also be related to its harmful effects
108 (Alonso-Magdalena et al., 2012). More recently, the chemicals used for flame
109 retardation, such as polybrominated diphenyl ethers (BDEs), have been reported to
110 impair brain development by affecting thyroid-related and estrogenic cellular
111 pathways (Zhou et al., 2002; Meerts et al., 2001). Therefore, the use of a certain type
112 of penta-BDE and octa-BDE technical formulations such as BDE-47 and BDE-99 has
113 been strictly regulated in many countries (European Parliament, 2002) and
114 decabromodiphenyl ether was recently listed as persistent organic pollutants under the
115 Stockholm Convention. Other than brominated flame retardants, phosphate flame

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116 retardants are used worldwide (Van der Veen et al., 2012). However, the evaluation of
117 possible endocrine-disrupting actions of phosphate flame retardants is less advanced
118 compared to that of brominated flame retardants. Recently, one of the co-authors of
119 this study conducted a study demonstrating that tris(2,6-dimethylphenyl) phosphate
120 (TDMPP), also known as 2,6-TXP, exerts estrogenic action at a level corresponding
121 to about 1/10,000 of that of estradiol, by *in vitro* reporter assay (Suzuki et al, 2013).
122 Since TDMPP is an impurity in flame retardant formulations of resorcinol
123 bis[di(2,6-dimethylphenyl) phosphate] (PBDMPP) (Matsukami et al. 2015), whose
124 demand has been increasing by the repressive trend of usage of BDEs, the possibility
125 to be environmentally exposed to TDMPP will predictably increase. On the other
126 hand, no study has been conducted whether TDMPP reveals endocrine-disrupting
127 action *in vivo*.

128 In this study, we evaluated whether TDMPP impairs sexual differentiation of the
129 brain using a mouse model. Moreover, to clarify whether this compound directly
130 enters the developing brain, we measured the level of TDMPP transferred to the brain
131 after maternal and neonatal injection. From the results of our toxicological and
132 exposure studies, we determined that TDMPP is a novel endocrine disruptor acting
133 directly on the mammalian brain.

134

135 Methods

136 Animals and developmental exposure toTDMPP

137 Pregnant C57BL/6J dams purchased from CLEA Japan (Tokyo, Japan) were used for
138 perinatal exposure to TDMPP. The day on which a vaginal plug was detected was
139 defined as gestational day (GD) 0. We prepared two experimental groups with
140 developmental exposure to TDMPP at different doses, to discover impairments
141 arising when perinatal mice were treated with TDMPP throughout the critical period
142 of brain sexual differentiation. From GD 14 to parturition, TDMPP (99.9%, Hayashi
143 Pure Chemical Ind., Ltd., Osaka, Japan), dissolved in sesame oil at the dose of 500
144 μg/0.2 ml sesame oil/day for the TDMPP-low dose group, and 5,000 μg/0.2 ml
145 sesame oil/day for the TDMPP-high dose group, was subcutaneously administered to
146 dams. On top of the prenatal exposure, pups from postnatal day (PND) 0 to 9 were
147 subcutaneously administered TDMPP at a dose of 50 μg/20 μl sesame oil/day for the
148 TDMPP-low group and 500 μg/20 μl sesame oil/day for the TDMPP-high group. A
149 vehicle control group (Oil group) was formed by administering an equivalent volume
150 of sesame oil to dams and pups on the same experimental schedule. To reduce stress
151 during treatments, we measured maternal and fetal body weight only at GD16 and
152 PND0, respectively. Thus, by using the body weights measured, we estimated daily
153 exposure levels of TDMPP-low and high dose groups. The maternal and fetal
154 exposure level of TDMPP-low group was estimated to be 15 mg/kg bw/day and 38
155 mg/kg bw/day, respectively, and those of TDMPP-high group was estimated to be
156 146 mg/kg bw/day and 384 mg/kg bw/day, respectively. To compare the effects of
157 TDMPP exposure to those of estrogen exposure, positive control groups were
158 established by administering 17β-estradiol (E_2, ≥ 98%, Sigma-Aldrich, St. Louis, MO,
159 USA) dissolved in sesame oil at the dose of 0.5 μg/0.2 ml sesame oil/day for the
160 E_2-low group and 2 μg/0.2 ml sesame oil/day for the E_2-high group, by subcutaneous
161 injections to dams from GD 14 to parturition. As for TDMPP exposure, subcutaneous
162 injections to pups from PND 0 to 9 were performed at the dose of 0.05 μg/20 μl
163 sesame oil/day for the E_2-low group, and 0.2 μg/20 μl sesame oil/day for the E_2-high

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164 group. The doses of E_2 were determined based on a previous report examining the
165 relative action of TDMPP compared to estrogen on ER-α by *in vitro* CALUX assay
166 (Suzuki et al., 2013): The action of the dose used in the E_2-low group should be
167 theoretically equivalent to the action of the dose used for the TDMPP-high group.
168 Thus, the relative level of putative estrogenic action on ER-α in the five groups is the
169 following: E_2-high > E_2-low ≈ TDMPP-high > TDMPP-low > Oil (vehicle control).
170 Litters were weaned from their mothers on PND 21 and housed with same-sex
171 littermates. Throughout the study, mice were housed in a room maintained at constant
172 temperature (24 ± 1ºC) and humidity (50 ± 10%) with a 12/12-h light/dark cycle.
173 Food and water were provided *ad libitum*. The administration of TDMPP to sexually
174 mature females was also performed in order to investigate whether TDMPP affects
175 sexual receptivity in adults. The relevant methods and results are described in the
176 Supplemental methods and results.
177 All procedures were approved by the Animal Care and Use Committee at the NIES
178 and conducted in strict accordance with the NIES guidelines. All efforts were made to
179 minimize the number of animals and their suffering.
180
181 **Transfer to brain**
182 To determine the transfer of TDMPP to the fetal and neonatal brain, pregnant
183 C57BL/6J females were purchased from CLEA Japan (Tokyo, Japan). Fifteen dams
184 were subcutaneously injected with TDMPP (5,000 μg/0.2 ml sesame oil) on GD 16.
185 Dams were sacrificed by decapitation and the brain and blood of the dams and the
186 fetuses (1 to 3 of each sex per dam) were collected at the time points of 0, 8, 16, 24,
187 and 48 h after injection (3 dams per time point). Pups born to five dams were
188 subcutaneously injected with TDMPP (500 μg/20 μl sesame oil) on PND 1 and
189 sacrificed by decapitation at the time points of 0, 8, 16, 24, and 48 h after injection (3
190 males from 3 dams and 3 females from 3 dams per time point), and brains were
191 collected. Samples were immediately frozen in dry-ice and stored at -75 ºC.
192
193 **Examination of general reproductive physiology and histology**
194 After birth, all the pups were subjected to body weight (BW) and anogenital distance
195 (AGD) measurements. The body weight was also measured at the time of weaning
196 (PND 21) and at 10 weeks of age. The AGD was also measured at the time of
197 weaning. All females were inspected daily for their first vaginal opening starting from
198 PND 18 until the opening was observed. Vaginal smears were taken daily for 26 days
199 starting from 9 weeks of age. Vaginal lavages were collected using 10 μl pipette tips
200 thinly wrapped with cotton moistened with deionized water. The lavages were placed
201 on a slide glass, air-dried and stained with 0.1% methylene blue solution. The estrous
202 stage of each individual on each day was determined based on the criteria described in
203 Cora et al. (2015), in which the estrous cycle is divided into 5 stages as follows:
204 proestrus (P), estrus (E), metestrus-1 (M1), metestrus-2 (M2), and diestrus (D). When
205 female mice reached 14 weeks of age, the ovaries were bilaterally removed under
206 isoflurane anesthesia from selected mice and weighed. The ratio of ovarian weight to
207 body weight was compared between groups. Ovary histology was examined by
208 paraffin sectioning and conventional hematoxylin-eosin staining.
209
210 **Overall scheme of the behavioral test battery**
211 When males and females reached 10 weeks and 14 weeks of age, respectively, one or
212 two mice of each sex were randomly selected from each litter, separated from their
213 littermates and housed individually in plastic cages (5 × 22 × 12 cm). At the time of

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214 isolation, selected females were ovariectomized under isoflurane anesthesia. They
215 were subjected to a behavioral test battery for emotional and socio-sexual behaviors,
216 consisting of open field test, light-dark transition test, and sexual behavior test for
217 both sexes, and aggressive behavior test for males only. All behavioral tests were
218 performed during the dark phase, starting more than 2 h after lights off. After
219 completing the behavioral tests, mice were sacrificed and blood and brain samples
220 were collected for enzyme immunoassays and immunohistochemistry. To eliminate
221 the litter effect, data collected from individuals were first averaged within littermates
222 of the same sex. Thus, the data shown in this study represent the mean value of the
223 score per litter, unless otherwise specified. The experimental design is shown in
224 Figure 1.
225
226 **Open field test**
227 Seven to 9 days after isolation, each mouse was tested in an open field apparatus (60
228 × 60 cm with 30 cm tall opaque walls) illuminated by white light (50 lux) twice in
229 two consecutive days, each trial lasting 10 min. The floor of the apparatus was
230 virtually divided into 25 square sections (12 × 12 cm each) and 9 inner squares were
231 designated as the center area. At the beginning of each trial, the mouse was placed in
232 a fixed corner, with the head facing the corner. Total moving distance (total distance)
233 and time spent in the center area (center time) were measured digitally by an
234 automated video tracking system (ANY-maze, Stoelting, USA).
235
236 **Light-dark transition test**
237 Five to 7 days after the open field test, each mouse was tested in a light-dark
238 transition test apparatus for 10 min. The test apparatus consisted of an enclosed dark
239 and an open-top light compartment (30 × 30 × 30 cm each), connected by an inner
240 doorway (3 × 3 cm) located in the center of the partition at the floor level. The
241 open-top light compartment was brightly illuminated with white light (350 lux). At
242 the beginning of the trial, the mouse was introduced in the dark compartment. The
243 latency to enter the light compartment and the cumulative time spent in the light
244 compartment were measured by an automated video tracking system (ANY-maze,
245 Stoelting, USA) (Sano et al., 2016).
246
247 **Male socio-sexual behavior tests**
248 Starting 5 to 7 days after the light-dark transition test, male mice were tested biweekly
249 for sexual and aggressive behaviors. All tests were performed under red light
250 illumination and videotaped. In the male sexual behavior test, each male was tested in
251 its home cage for sexual behavior meant to lure female C57BL/6J mice. All lured
252 females had been ovariectomized and primed with subcutaneous injections of
253 estradiol benzoate (10 μg/0.1 ml dissolved in sesame oil) twice at 48 and 24 h before
254 testing and progesterone (500 μg/ 0.1 ml dissolved in sesame oil) once at 4-6 h before
255 testing to ensure high sexual receptivity. Three trials were performed, each lasting 30
256 min. The number of attempted mounts, mounts, and intromissions was scored for each
257 mouse (Sano et al., 2016).
258 In the male aggressive behavior test, each mouse was tested in a resident-intruder
259 paradigm against olfactory bulbectomized male C57BL/6J mice. On each trial week,
260 the mice were tested on three consecutive days, for a total of 9 trials, each lasting 15
261 min. The number and duration of aggressive bouts toward the intruder were scored for
262 each mouse. The data from the 3 trials performed each week were averaged for each
263 mouse and used for statistical analysis. An aggressive bout was defined as a set of

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264 behavioral interactions that included at least one of the following actions: Chasing,
265 boxing, wrestling, biting, tail-rattling, and offensive lateral attack. If the interval
266 between 2 aggressive bouts did not exceed 3 seconds, the 2 bouts were considered to
267 be continuous and scored as 1 bout (Sano et al., 2016).
268
269 **Female sexual behavior test**
270 Five to 7 days after the light-dark transition test, female mice were tested for sexual
271 behavior toward a sexually experienced ICR/JCL male mouse in the male's home
272 cage. The test was performed weekly for a total of 5 trials. Female mice were tested
273 under an artificial estrous condition in which they had been primed with subcutaneous
274 injection of estradiol benzoate (5 µg dissolved in 0.1 ml sesame oil) twice at 48 and
275 24 h before testing, and progesterone (250 µg dissolved in 0.1 ml sesame oil) once at
276 4-6 h before testing. Each test lasted until females received either 15 mounts or 15
277 intromissions. The number of lordosis responses to either mount or intromission was
278 scored for each mouse. A lordosis quotient was calculated by dividing the number of
279 lordosis responses by the 15 mounts or intromissions (Sano et al., 2016).
280
281 **Blood and brain sampling**
282 After the completion of the behavioral testing, mice were deeply anesthetized with a
283 solution of a 1:1 mixture of sodium pentobarbital (60 mg/kg BW) and heparin (1,000
284 units/ml), and blood was collected from the left ventricle. They were then
285 transcardially perfused with 0.1 M phosphate-buffer (PBS; pH 7.2), followed by 4%
286 paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed, post-fixed with 4%
287 PFA in 0.1 M PBS overnight at 4ºC, and cryoprotected in 0.1 M PBS containing 30%
288 sucrose. Sections (30 µm thick) were made on a freezing microtome (REM-710,
289 Yamato, Japan) at 120 µm intervals. Male and female brains of the Oil, TDMPP-low,
290 TDMPP-high and E_2-low groups were used for immunohistochemistry to detect
291 sexually dimorphic nuclei.
292
293 **Enzyme immunoassay for testosterone and estradiol**
294 Samples were extracted from male plasma (100 µl) with ethyl acetate. Testosterone
295 and estradiol concentrations were determined using enzyme immunoassay kits for
296 each hormone (Cayman Chemicals, Ann Arbor, MI, USA) according to the
297 manufacturer's instructions.
298
299 **Calbindin D-28K immunohistochemistry to detect sexual dimorphic nuclei**
300 Endogenous peroxidase in the brain section was removed by incubating with 0.6%
301 H_2O_2 containing 0.05 M PBS with 1% Triton X-100 (PBST) for 60 min at room
302 temperature. The sections were treated with 5% normal goat serum containing PBST
303 for 60 min at room temperature to prevent the nonspecific binding of the antibody.
304 Afterward, they were incubated with a monoclonal antibody against CB (C9848,
305 Sigma Aldrich, St. Louis, MO, USA, 1:15,000) for 2 days at 4ºC. Subsequently, the
306 sections were rinsed with PBST and incubated in a peroxidase-labeled polymer
307 conjugated with goat anti-mouse immunoglobulin (Dako Envision Plus, Dako,
308 Carpinteria, CA, USA) for 30 min at room temperature. After rinsing again with
309 PBST, the sections were stained with 3,3'-diaminobenzidine in chromogen solution
310 (Dako). Finally, they were mounted on gelatin-coated slides.
311
312 **Delineation of sexually dimorphic nuclei**

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313 CB is a protein maker for detecting two specific sexually dimorphic nuclei: The
314 calbindin-sexually dimorphic nucleus (Calb-SDN), subregion of the medial preoptic
315 area; and the principal nucleus of the bed nuclei of the stria terminalis (BNSTp)
316 (Budefeld et al. 2008, Gilmore et al. 2012, Orikasa & Sakuma 2010, Sickel &
317 McCarthy 2000). We defined the Calb-SDN as the distinctive ellipsoidal cluster of
318 CB-immunoreactive cells at the preoptic area/anterior hypothalamus, dorsolaterally
319 angled from the third ventricle, and located dorsal to the optic chiasm, lateral to the
320 third ventricle, and ventral to the BNSTp (Gilmore et al, 2012). We also defined the
321 BNSTp as the clusters of CB-immunoreactive cells between the stria terminalis and
322 the stria medullaris of the thalamus, in the area surrounded by the lateral ventricle and
323 the third ventricle (Gilmore et al. 2012, Moe et al. 2016, Wittmann & Mclennan
324 2013).
325
326 **Stereological analysis of sexually dimorphic nuclei**
327 The CB-stained sections were observed under the light microscope. The volume and
328 number of CB-immunoreactive (CB-ir) cells in Calb-SDN and BNSTp were analyzed
329 using the Stereo Investigator software (MBF Bioscience Inc., Williston, VT, USA).
330 Since each slide was randomly assigned an identification number not related to the
331 original number of the animal, the observer who performed the analysis was blinded
332 to the sample origin. The optical fractionator method of the stereological probe
333 workflow in the software was used to analyze the CB-stained sections. The outlines of
334 Calb-SDN and BNSTp were traced on the left side of brain sections to determine the
335 analysis area according to a mouse brain atlas (Paxinos & Franklin, 2004). The CB-ir
336 cells were counted in a defined counting frame and grid for each area. Details on the
337 analysis are reported in Table 1.
338
339 **Chemical analysis of TDMPP and its metabolites in brain**
340 After thawing of the brain samples, tris(3,5-dimethylphenyl-d9) phosphate (Hayashi
341 Pure Chemical Ind., Ltd.) was added as an internal standard, and the samples were
342 homogenized by an ultrasonic homogenization device and extracted with methanol.
343 The crude extract was passed through the Oasis Wax (150 mg/30 μm) cartridge
344 column (prewashed with 5 ml of methanol). Two fractions were collected: 3 mL of
345 methanol (fraction 1) and 5 mL of 0.5% ammonium hydroxide in methanol (fraction
346 2). The eluate of fraction 1 was passed through the ENVI-Carb 250 mg cartridge
347 column (prewashed with 5 ml of dichloromethane:toluene (3:1, v/v) mixture). 3 ml of
348 dichloromethane:toluene (3:1, v/v) mixture was passed through the ENVI-Carb
349 column and the eluate was collected (fraction 1A). The eluates of fraction 1A and 2
350 were evaporated and redissolved in 0.5 ml of methanol. An electrospray
351 ionization-quadrupole time-of-flight mass spectrometer (ESI-QTOF-MS) equipped
352 with an ultra-high-performance liquid chromatograph (LC) system (1290
353 Infinity/6530 Accurate-Mass QTOF LC/MS system; Agilent Technologies Inc., Santa
354 Clara, CA, USA) with a reversed-phase LC column (ZORBAX Eclipse Plus C18
355 RRHD, 50 mm × 2.1 mm i.d., 1.8 mm; Agilent Technologies Inc.) was used for the
356 identification and quantification of TDMPP and its metabolites. The mass range for
357 the MS investigation was set at m/z 100–1500. The mass range for MS/MS
358 investigation was set to m/z 50–1000. The inter- and intra-day variation of
359 measurements were 14% and 12%, respectively, and the mean recovery rate of
360 internal standard from fetal and neonatal brains was 75%.
361
362 **Statistical Analysis**

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363 All data are presented as the mean ± standard error of the mean (SEM). Data were
364 analyzed using analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests
365 or Student t-test. Differences were considered statistically significant at p-values less
366 than 0.05. The analysis was performed with the SPSS 19.0 statistical package (SPSS
367 Inc., Chicago, IL, USA).
368
369

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370 **Results**

371 **Body weight**

372 No statistically significant effect of TDMPP exposure on body weight of either males
373 or females at birth, PND 21, or 10 weeks of age was found (Figure 2A-F). In addition,
374 there was no significant difference in body weight between the control Oil group and
375 the E_2-treated groups, whereas a slight but significant difference was found among the
376 E_2-treated groups. A detailed description of the statistical analysis is reported in the
377 Supplementary Results.
378

379 **Anogenital distance**

380 A statistically significant effect of TDMPP exposure on AGD was found by ANOVA
381 among the male groups at birth and PND 21, but we could not detect which groups
382 caused the significant difference, at least by Bonferroni *post hoc* test (Figure 3A-D).
383 There was no significant difference among the female groups at birth and PND 21. A
384 detailed description of the statistical analysis is reported in the Supplemental results.
385

386 **Female reproductive physiology and histology**

387 In the TDMPP-high and E_2-high groups, the day we first observed vaginal opening
388 was significantly earlier compared to the Oil, E_2-low, and TDMPP-low groups
389 [$F(4,17) = 20.132$, $P < 0.001$; Bonferroni *post hoc* test: $P < 0.001$, TDMPP-high and
390 E_2-high vs. Oil or TDMPP-low; $P = 0.011$, TDMPP-high vs. E_2-low; $P = 0.018$,
391 E_2-high vs. E_2-low; Figure 4A,B]. The estrous cycle, detected by vaginal smear after
392 sexual maturation, was impaired in both the TDMPP and E_2-exposed groups (Figure
393 5A-E). The number of estrous days in the TDMPP-low, TDMPP-high, E_2-low and
394 E_2-high groups was significantly reduced compared to the Oil group [$F(4,24) =$
395 25.228, $P < 0.001$; Bonferroni *post hoc* test: $P < 0.001$, TDMPP-high, E_2-low, and
396 E_2-high vs. Oil; $P = 0.011$, TDMPP-low vs. Oil; Figure 5F]. No difference was found
397 in the ratio of ovarian weight to body weight between the groups [$F(4,24) = 2.211$, P
398 $= 0.098$; Figure 6].
399

400 **Open field test**

401 In males, no difference was found in total moving distance or center time between the
402 groups [Figure 7A and B]. In females, there was no significant difference in total
403 moving distance or center time between the Oil group and other groups, whereas there
404 was a significant difference in total moving distance between the E_2-treated groups
405 and the TDMPP-low group [Figure 7C and D]. A detailed description of the statistical
406 analysis is reported in the Supplemental results.
407

408 **Light-dark transition test**

409 There was no difference in the time spent in the light compartment between the Oil
410 group and the TDMPP-treated groups in males, whereas the male mice in the E_2-high
411 group spent significantly shorter time in the light compartment than the mice in other
412 male groups [$F(4,25) = 7.672$, $P < 0.001$; Bonferroni *post hoc* test: $P = 0.001$, E_2-high
413 vs. Oil or TDMPP-high; $P = 0.002$, E_2-high vs. E_2-low; $P = 0.030$, E_2-high vs.
414 TDMPP-low; Figure 8A]. The latency to enter the light compartment did not differ
415 among the groups [$F(4,25) = 1.739$, P = 0.173; Figure 8B]. In females, no differences
416 were found among the groups in the time spent in the light compartment [$F(4,24) =$
417 2.292, $P = 0.089$; Figure 8C] or the latency to enter the light compartment [$F(4,24) =$
418 0.306, $P = 0.871$; Figure 8D].
419

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420 **Male socio-sexual behavior tests**
421 Regarding male sexual behavior, no differences between the Oil group and the
422 TDMPP-treated groups were found in the number of attempted mounts [treatment,
423 $F(4,25) = 0.476$, $P = 0.753$; treatment × test number, $F(8,50) = 1.259$, $P = 0.286$;
424 Figure 9A], mounts and intromissions [treatment, $F(4,25) = 1.226$, $P = 0.325$;
425 treatment × test number, $F(8,50) = 0.441$, $P = 0.890$; Figure 9C]. No differences were
426 found between the Oil group and the E_2-treated groups either, although the mice in the
427 E_2-high group showed significantly higher number of mounts compared to the E_2-low
428 group [treatment: $F(4,25) = 2.831$, $P = 0.046$; Bonferroni *post hoc* test: $P = 0.050$,
429 E_2-high vs. E_2-low; treatment × day: $F(4,25) = 1.519$, $P = 0.174$; Figure 9B]. As for
430 aggressive behavior, the mice in the TDMPP-high group showed significantly higher
431 number of aggressive bouts compared to the Oil, TDMPP-low and E_2-low groups
432 [treatment: $F(4,25) = 6.403$, $P = 0.001$; Bonferroni *post hoc* test: $P = 0.005$,
433 TDMPP-high vs. Oil; $P = 0.002$, TDMPP-high vs. TDMPP-low; $P = 0.030$,
434 TDMPP-high vs. E_2-low; treatment × day: $F(8,50) = 0.575$, $P = 0.793$; Figure 10A].
435 A similar tendency was observed in the total duration of aggressive bouts, but the
436 difference was not statistically significant [treatment: $F(4,25) = 2.158$, $P = 0.103$;
437 treatment × day: $F(8,50) = 1.147$, $P = 0.350$; Figure 10B].
438
439 **Enzyme immunoassay for plasma testosterone and estradiol**
440 No differences among the male groups were found in plasma testosterone levels
441 [treatment: $F(4,25) = 1.098$, $P = 0.379$, Figure 11A]. No differences in plasma
442 estradiol concentration between the Oil and any other group were found, whereas
443 males in the E_2-high group showed significantly higher concentrations compared to
444 the TDMPP-low, TDMPP-high, and E_2-low groups [treatment: $F(4,25) = 5.725$, $P =$
445 0.002; Bonferroni *post hoc* test: $P = 0.003$, E_2-high vs. TDMPP-high or E_2-low; $P =$
446 0.027, E_2-high vs. TDMPP-low; Figure11B].
447
448 **Female sexual behavior**
449 The lordosis quotient, an index of sexual receptivity, in the mice of the TDMPP-high,
450 E_2-low and E_2-high groups was significantly reduced compared to that of the Oil
451 group [treatment: $F(4,24) = 6.822$, $P = 0.001$; Bonferroni *post hoc* test, $P = 0.001$,
452 TDMPP-high vs. Oil; $P = 0.008$, E_2-low vs. Oil; $P = 0.043$, E_2-high vs. Oil; treatment
453 × day: $F(16,96) = 1.228$, $P = 0.261$; Figure 12], demonstrating that the
454 endocrine-disrupting action of the exposure to either E_2 or TDMPP during the critical
455 period of brain sexual differentiation impairs the development of sexual receptive
456 behavior.
457
458 **Analysis of sexually dimorphic nuclei**
459 Calb-SDN volume and cell number were significantly higher in the male than in the
460 female Oil group (Student t-test: $P < 0.001$, Figure 13A, B, C, and D). In females,
461 Calb-SDN volume and cell number in the TDMPP-low, TDMPP-high and E_2-low
462 groups were significantly higher than in the Oil group (ANOVA: volume: $F(3,15) =$
463 22.734, $P < 0.001$; Bonferroni *post hoc* test: $P < 0.001$, TDMPP-low and
464 TDMPP-high vs. Oil, P = 0.001, E_2-low vs. Oil, number: $F(3,15) = 19.012$, $P < 0.001$;
465 Bonferroni *post hoc* test: $P < 0.001$, TDMPP-low, TDMPP-high and E_2-low vs. Oil,
466 Figure 13B and D). In males, Calb-SDN volume and cell number of the TDMPP-low
467 group were significantly higher than the Oil group, but there was no significant
468 difference among the Oil, TDMPP-high and E_2-low groups (ANOVA: volume:
469 $F(3,14) = 14.372$, $P < 0.001$; Bonferroni *post hoc* test: $P < 0.001$, TDMPP-low vs. Oil,

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470 number: $F(3,14) = 4.678$, $P = 0.018$; Bonferroni *post hoc* test: $P = 0.021$,
471 TDMPP-low vs. Oil Figure 13A and C).
472 Similarly, BNSTp volume and cell number were significantly higher in the male than
473 in the female Oil group (Student t-test: $P = 0.001$, Figure 13E,F,G and H). BNSTp
474 cell number in the females of the TDMPP-high groups were significantly higher than
475 in the Oil group (ANOVA: $F(3,15) = 5.369$, $P = 0.009$; Bonferroni *post hoc* test: $P =$
476 0.009, TDMPP-high vs. Oil, Figure 13H), whereas no difference was found in the
477 volume (ANOVA: $F(3,16) = 1.221$, $P = 0.334$, Figure 13F). In males, there was no
478 difference in either BNSTp volume or cell number between groups (ANOVA:
479 volume: $F(3,14) = 0.871$, $P = 0.480$, number: $F(3,14) = 0.216$, $P = 0.884$, Figure 13E
480 and G).
481
482 **<u>Concentrations of TDMPP and its metabolites in brain</u>**
483 Using MS and MS/MS, we first detected TDMPP and its metabolites in neonatal
484 (PND 1) brain samples 16 h after treatment with TDMPP, based on precise molecular
485 weight information about TDMPP and its metabolites and related ions. We detected
486 TDMPP and 4 different metabolites, denoted by di(2,6-dimethylphenyl) phosphate
487 (DDMPP), TDMPP-M1, TDMPP-M2-1, and TDMPP-M2-2. From the molecular
488 structure of such metabolites, a number of conclusions could be drawn: (1) DDMPP is
489 a hydroxylation metabolite of TDMPP; (2) TDMPP-M2-1 and TDMPP-M2-2 are
490 oxidation metabolites of TDMPP; and (3) TDMPP-M1 is an oxidation metabolite of
491 DDMPP, or a hydroxylation metabolite of TDMPP-M2-1 or TDMPP-M2-2
492 (Supplemental figure 1). We measured the levels of TDMPP and its metabolites in
493 fetal (GD 16) and neonatal (PND 1) brain samples at 0, 8, 16, 24 and 48 h after
494 treatment with TDMPP. In the case of fetal brain samples, TDMPP levels rose to 70
495 ng/g 8 h after treatment, corresponding to 0.000070% of the treatment dose (5,000
496 μg). In the case of neonatal brain samples, TDMPP and its metabolites were detected
497 8 h after treatment, and the TDMPP level rose to 870 ng/g 16 h after treatment,
498 corresponding to 0.017% of the treatment dose (500 μg), suggesting that the relative
499 accumulation of TDMPP is higher in the neonatal than in the fetal brain. In the fetal
500 brain, TDMPP was also still detectable at 16, 24, and 48 h, and its levels were 34%,
501 93%, and 28% of the 8-hour level, respectively. In the neonatal brain, TDMPP was
502 still detectable at 24 and 48 h, with levels corresponding to 27% and 16% of that at 16
503 h, respectively (Supplemental table 1 and 2).
504
505
506

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507 **Discussion**

508

509 In this study, we evaluated the endocrine-disrupting action of TDMPP, an impurity in
510 flame retardant formulations of PBDMPP not recognized as an endocrine disruptor,
511 although the acute and chronic toxicities of TDMPP have already been evaluated
512 (Van der Veen & de Boer, 2012). However, a recent *in vitro* screening of
513 representative phosphate flame retardants and related compounds found the activation
514 of ER-α by TDMPP to be at a level corresponding to about 1/10,000 of that of
515 estradiol. This activation is the highest among 23 chemicals examined in the study,
516 and the activation potency of TDMPP is considered to be similar to that of
517 bisphenol-A, reported in previous literature (Suzuki et al., 2013). Thus, we judged
518 that the endocrine-disrupting action of TDMPP exposure should be preferentially
519 examined compared to other phosphate flame retardants and related chemicals. To
520 evaluate the estrogenic activity of TDMPP, we decided to use a mouse model to
521 specifically investigate the impairment in brain sexual differentiation. The influence
522 of chemicals having estrogenic activity, such as diethylstilbestrol, bisphenol-A, and a
523 certain type of flavonoids, is known to disturb the brain sexual differentiation when
524 administered during either late pregnancy or the neonatal period, i.e. the critical
525 periods of brain differentiation in rodents (Mendes, 2002; Vandenberg et al., 2009).
526 Therefore, we administered TDMPP, and E_2 as a positive control, to mice throughout
527 the critical period and examined the behavioral, physiological and histological
528 changes related to reproduction.
529 Given that no significant differences in body weight and AGD during development
530 in the TDMPP-treated groups were found, gross morphological effects could not be
531 detected, at least with the treatment schedule we used. However, we cannot exclude
532 the possibility that TDMPP affects the morphogenesis of estrogen-sensitive organs,
533 because it has been reported that estrogenic agents in general affect AGD when
534 administered at earlier time points (Honma et al., 2002). On the other hand, we could
535 detect impairments in different aspects of female physiology and behavior due to
536 developmental exposure to TDMPP, although the open field and light-dark transition
537 tests were not altered. Acceleration of vaginal opening, an index of precocious
538 puberty, and irregular estrous cycle, an index of impairment in the
539 hypothalamo-pituitary-gonadal axis, are known to be typical effects of exposure to
540 estrogenic chemicals of rodents in the critical period of brain sexual differentiation:
541 Our results obtained in both TDMPP- and E_2-treated females were in agreement with
542 the reported impairments in animals developmentally exposed to estrogenic chemicals.
543 Similarly, sexual receptivity in females, examined by the lordosis behavior test, was
544 impaired by developmental exposure to TDMPP. Since these physiological and
545 behavioral changes mostly coincided with those due to exposure to the positive
546 control E_2, we conclude that the effect of TDMPP on female reproductive function
547 impairment is mediated by the activation of the estrogen signaling pathways, as
548 expected.
549 In rodents, the differentiation of core sexual behavior and reproductive physiology is
550 thought to be influenced by exposure to gonadal hormones, whereas the sexual
551 differentiation of various other aspects of physiology and behavior, such as social
552 communication, is thought to be determined at least in part by the interaction of
553 gonadal hormones and sex chromosomes (Arnold, 2004; McCarthy & Arnold, 2011,
554 Maekawa et al., 2014). In particular, sexual and aggressive behaviors are thought to
555 be masculinized and de-feminized by the exposure to testosterone secreted from testes
556 during the critical period of brain sexual differentiation (Bronson & Desjardins, 1968;

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557 Pfaff & Zigmond, 1971). Testosterone that enters the brain can be converted into E_2
558 by brain aromatase, and the activation of estrogen receptors by locally synthesized
559 brain E_2 mediates the process of brain masculinization and de-feminization (Naftolin
560 et al., 1971; McEwen et al., 1977). Conversely, the lack of exposure to testosterone
561 and/or estrogen during the critical period of sexual differentiation is essential for the
562 differentiation of female receptive behavior. Indeed, the exogenous injection of either
563 testosterone or estrogen during the critical period of sexual differentiation was
564 reported to impair the development of sexual behavior in females (McDonald &
565 Doughty, 1972; Kouki et al., 2003; Kanaya & Yamanouchi, 2012). Furthermore, since
566 loss-of-function in estrogen signaling by ER-α, but not ER-β, gene knockout impairs
567 masculinization and de-feminization (Ogawa et al., 1998, 1999), the process of
568 differentiation of female receptive behavior and reproductive physiology is
569 predominantly mediated via the ER-α pathway. In order to know whether ER-α in the
570 brain is "directly" involved in the physiological and reproductive functions, we
571 devised various experimental approaches. As a first approach, through chemical
572 exposure measurements, we confirmed the direct transfer of the injected TDMPP and
573 its metabolites to both the fetal and neonatal brain. As a second approach, through
574 histological techniques, we examined the effect of TDMPP on the formation of the
575 typical male-dominant sexual dimorphic nuclei, finding that the volume and cell
576 number of the Calb-SDN and BNSTp were increased up to the level of male mice by
577 the developmental exposure of females to TDMPP. It has been reported that the
578 treatment with an ER-α, but not ER-β, agonist mimics the effect of E_2 on the
579 establishment of sexual dimorphism in the sexually dimorphic nucleus of the preoptic
580 area, including the Calb-SDN (Patchev et al., 2004; Tsukahara 2009; Sickel &
581 McCarthy, 2000). It has also been reported that the volume and number of neurons in
582 the BNSTp were feminized in male mice deficient in the ER-α gene (Tsukahara et al.,
583 2011). These reports demonstrate that cellular signaling downstream of ER-α is
584 required for the formation of these nuclei. Taken together, these previous reports and
585 our experiments show that the activation of ER-α by TDMPP in the brain could be a
586 most likely mechanism explaining the behavioral and physiological changes in female
587 mice.
588 Concerning male behavior, the mice in the TDMPP-high group revealed higher
589 aggressive behavior compared to those in the control Oil group, whereas there was no
590 difference in male sexual behavior among groups. This behavioral change might
591 reflect the impact of brain hyper-masculinization by developmental exposure to
592 TDMPP. In terms of hormonal effects, male aggressive behavior is known to be
593 regulated by brain sexual differentiation in the critical period, and sex steroid
594 hormonal levels in the adult (Bronson & Desjardins, 1968). Since testosterone and
595 estradiol levels in the adults of the TDMPP-high groups were similar to those of the
596 Oil control group, brain sexual differentiation during the developmental period might
597 lead to higher aggressiveness. We also examined the volume and cell number of
598 sexual dimorphic nuclei in the male groups, but no significant change was appreciated
599 in either Calb-SDN or BNSTp in the TDMPP-high group, although the volume and
600 cell number of Calb-SDN were rather significantly increased in the TDMPP-low
601 group. Therefore, we cannot find a clear histological change corresponding to the
602 higher aggression shown by the TDMPP-high group. To summarize the results on
603 male mice, the effect of TDMPP was limited compared to females, and the group
604 revealing behavioral abnormality was not consistent with that revealing histological
605 abnormality. Since the male brain is naturally formed under developmental exposure

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606 to estradiol converted from testosterone within the brain, the additional estrogenic
607 effect of TDMPP might be less pronounced in terms of activation of ER-α.
608 The fertility rate of mating pairs in each group was also examined (Supplemental
609 Table 1). Mating pairs of the TDMPP-low group showed low fertility, while all
610 mating pairs of the TDMPP-high group were infertile. These results clearly
611 demonstrate that TDMPP exposure causes lower birth rate, presumably due to the
612 lowered reproductive behavior and physiology in females. Usage of organophosphate
613 flame retardants has recently increased in many household products, and the transfer
614 of organophosphate flame retardants to house dust in living situations has been
615 reported worldwide (Stapleton et al., 2009). Thus, not only industrial but also
616 environmental exposure to TDMPP will be increased with the increasing use of
617 organophosphorus phosphate flame retardants in the future. Based on our empirical
618 data, the contamination of TDMPP in household products, and the subsequent home
619 exposure to TDMPP during pregnancy and/or the neonatal period could be suspected
620 to endanger women's health in the next generation. Norms limiting the contamination
621 of TDMPP in products are thus required.
622
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808 **Figure legends**
809
810 **Figure 1: The experimental design of the developmental exposure and behavioral**
811 **test battery.**
812
813 **Figure 2: Effect of developmental TDMPP exposure on body weight. (A-C)** male
814 and **(D-F)** female mice of each treatment group. The body weight at birth **(A,D)**, PND
815 21 **(B,E)**, and 10 weeks of age **(C,F)**. The number in parentheses indicate number of
816 liters. The data are presented as the mean ± SEM. **$P < 0.01$.
817
818 **Figure 3: Effect of developmental TDMPP exposure on anogenital distance.**
819 **(A,B)** male and **(C,D)** female of each treatment group. The anogenital distance at
820 birth **(A,C)** and PND 21 **(B,D)**. The number in parentheses indicate number of liters.
821 The data are presented as the mean ± SEM.
822
823 **Figure 4: Effect of developmental TDMPP exposure on first vaginal opening. (A)**
824 Percentage of females displayed vaginal opening. (B) Average ages of first vaginal
825 opening. The number in parentheses indicate number of liters. The data are presented
826 as the mean ± SEM. **$P < 0.01$ vs Oil or TDMPP-low. *$P < 0.05$ vs E_2-low.
827
828 **Figure 5: Effect of developmental TDMPP exposure on estrous cycle. (A-E)** The
829 representative estrous cycle pattern of each treatment group. E = estrus, P = proestrus,
830 M1 = metestrus 1, M2 = metestrus 2, D = diestrus. **(F)** The number of cycles within
831 the 27 days of recording period. The number in parentheses indicate number of liters.
832 The data are presented as the mean ± SEM. **$P < 0.01$, *$P < 0.05$ vs Oil.
833
834 **Figure 6: Effect of developmental TDMPP exposure on the ratio of ovarian**
835 **weight to body weight.** The number in parentheses indicate number of liters. The
836 data are presented as the mean ± SEM.
837
838 **Figure 7: Effect of developmental TDMPP exposure on the open field activity.**
839 **(A,B)** male and **(C,D)** female of each treatment group. **(A,C)** the total moving
840 distance and **(B,D)** time spent in center area of open field test apparatus. The number
841 in parentheses indicate number of liters. The data are presented as the mean ± SEM.
842
843 **Figure 8: Effect of developmental TDMPP exposure on anxiety-related behavior,**
844 **as measured in the light-dark transition test. (A,B)** male and **(C,D)** female of each
845 treatment group. (A,C) the total time spent in, and (B,E) the latency to enter the light
846 compartment of the light-dark transition apparatus. The number in parentheses
847 indicate number of liters. The data are presented as the mean ± SEM. **$P < 0.01$ vs
848 Oil, TDMPP-high, or E_2-low, *$P < 0.05$ vs TDMPP-low.
849
850 **Figure 9: Effect of developmental TDMPP exposure on male sexual behavior.**
851 The total number of **(A)** attempted mount, **(B)** mount, and **(C)** intromission toward
852 female stimuli. The number in parentheses indicate number of liters. The data are
853 presented as the mean ± SEM. *$P < 0.05$.
854
855 **Figure 10: Effect of developmental TDMPP exposure on male aggressive**
856 **behavior. (A)** Total number and **(B)** the duration of aggressive bouts toward intruder

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857 stimuli. The number in parentheses indicate number of liters. The data are presented
858 as the mean ± SEM. $**P < 0.01$, $*P < 0.05$.
859
860 **Figure 11: Effect of developmental TDMPP exposure on plasma testosterone and**
861 **estradiol concentration in male.** Plasma concentration of **(A)** testosterone and **(B)**
862 estradiol. The number in parentheses indicate number of liters. The data are presented
863 as the mean ± SEM. $**P < 0.01$ vs TDMPP-high, or E_2-low, $*P < 0.05$ vs
864 TDMPP-low.
865
866 **Figure 12: Effect of developmental TDMPP exposure on female sexual behavior.**
867 The number in parentheses indicate number of liters. The data are presented as the
868 mean ± SEM. $**P < 0.01$, $*P < 0.05$ vs Oil.
869
870 **Figure 13: Effect of developmental TDMPP exposure on the sexually dimorphic**
871 **nuclei. (A,C,E,G)** male and **(B,D,F,H)** female of each treatment group. (A,B) The
872 volume and (C,D) number of cells in the Calb-SDN. (E,F) The volume and (G,H)
873 number of cells in the BNSTp. The number in parentheses indicate number of animals.
874 The data are presented as the mean ± SEM. $**P < 0.01$, $*P < 0.05$ vs Oil.
875
876
877

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878 **Supplementary methods**

879 **Effect of TDMPP on the induction of lordosis and uterine weight in sexually**
880 **mature females**

881 Female C57BL/6J mice purchased from CLEA Japan (Tokyo, Japan) were
882 ovariectomized at 11 weeks of age under isoflurane anesthesia. At 12 weeks of age
883 (Test 1), either 17β-estradiol (5 μg/2 ml sesame oil), TDMPP (99.9%, Hayashi Pure
884 Chemical Ltd, Osaka, Japan, 50 mg/2 ml sesame oil), or sesame oil (2 ml) were
885 subcutaneously injected twice, at 48 and 24 h before testing. Additionally,
886 progesterone (250 μg/0.1 ml sesame oil) was subcutaneously injected 4 h before
887 testing Females were tested for sexual behavior toward a sexually experienced
888 ICR/JCL male mouse in the male's home cage. Each test lasted until females received
889 either 15 mounts or 15 intromissions. The number of lordosis responses to either
890 mount or intromission was scored for each mouse. A lordosis quotient was calculated
891 by dividing the number of lordosis responses by 15 mounts or intromission. The same
892 test was also performed at 13 weeks of age (Test 2). Immediately after the last
893 lordosis test, females were sacrificed by decapitation and uterine weight was
894 measured.
895

896 **Reproduction rate in mating**

897 Four to five female-male pairs in the Oil, TDMPP-low, TDMPP-high and E_2-low
898 groups at 15 weeks of age were randomly selected and bred for three estrous cycles to
899 determine whether females showed vaginal plug. In addition, the number of pups was
900 counted if the female became pregnant and delivered.
901

902 **Ovarian morphology**

903 Ovaries at 14 weeks of age were fixed by 4% paraformaldehyde in 0.05 M PBS,
904 embedded in paraffin blocks and cut by microtome at a thickness of 10 μm. Sections
905 were stained by conventional hematoxylin-eosin staining and were observed by light
906 microscopy.
907
908

909 **Supplementary results**

910

911 **Body weight**

912 No significant effect of TDMPP exposure was found on the body weight of either
913 male or female mice at birth, PND 21 and 10 weeks of age (Figure 2A-F). On the
914 other hand, a slight but significant difference was found among the E_2 positive control
915 groups.
916 In male mice, there was no difference between the Oil and the other groups, whereas
917 there was a statistically significant difference at birth between the E_2-low and E_2-high
918 groups [$F(4,25) = 5.215$, $P = 0.003$; Bonferroni *post hoc* test: $P = 0.002$, E_2-low vs.
919 E_2-high; Figure 2A]. This difference between male groups disappeared at PND 21 and
920 10 weeks of age [PND 21: $F(4,25) = 1.451$, $P = 0.247$, Figure 2B; 10 weeks: $F(4,25)$
921 $= 1.154$, $P = 0.355$, Figure 2C]. Additionally, female mice in the E_2-high group were
922 significantly heavier than those in the E_2-low and TDMPP-low groups [$F(4,24) =$
923 5.526, $P = 0.003$; Bonferroni *post hoc* test: $P = 0.007$, E_2-high vs. E_2-low or $P = 0.004$,
924 E_2-high vs. TDMPP-low; Figure 2D], whereas there was no difference between the
925 Oil group and other groups. At PND 21, there was no difference in their body weight
926 [$F(4,24) = 1.097$, $P = 0.381$, Figure 2E]. At 10 weeks of age, although ANOVA
927 detected a significant treatment effect, no differences between groups were found in

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928 the Bonferroni *post hoc* test [$F(4,24) = 3.112$, $P = 0.034$; Bonferroni *post hoc* test, ns;
929 Figure 2F].
930
931 **Anogenital distance**
932 In male mice, although ANOVA detected a significant treatment effect on AGD at
933 birth, no differences between groups were revealed by Bonferroni *post hoc* test
934 [$F(4,25) = 3.508$, $P = 0.021$; Bonferroni *post hoc* test, ns; Figure 3A]. At PND 21,
935 AGD did not differ between groups in males [$F(4,25) = 2.065$, $P = 0.116$; Figure 3B].
936 In female mice, no differences were found in AGD among groups at birth or PND 21
937 [at birth: $F(4,24) = 1.022$, $P = 0.416$, Figure 3C; PND 21: $F(4,24) = 1.309$, $P = 0.295$,
938 Figure 3D].
939
940 **Open field test**
941 In males, no difference was found in total moving distance [treatment: $F(4,25) =$
942 0.689, $P = 0.606$; treatment × day: $F(4,25) = 2.264$, $P = 0.091$; Figure 7A] and center
943 time between groups [treatment: $F(4,25) = 0.812$, $P = 0.529$; treatment × day: $F(4,25)$
944 $= 1.278$, $P = 0.305$; Figure 7B]. Although female mice in the E_2-low and E_2-high
945 groups showed decreased total moving distance compared to the TDMPP-low group,
946 there was no difference between TDMPP-exposed groups and the Oil group
947 [treatment: $F(4,24) = 3.975$, $P = 0.013$; Bonferroni *post hoc* test: $P = 0.036$, E_2-low vs.
948 TDMPP-low; $P = 0.020$, E_2-high vs. TDMPP-low; treatment × day: $F(4,24) = 2.616$,
949 $P = 0.060$; Figure 7C]. There was no difference in center time among groups in
950 females [treatment: $F(4,24) = 1.201$, $P = 0.336$; treatment × day: $F(4,24) = 0.225$, $P =$
951 0.921; Figure 7D].
952
953 **Effect of TDMPP on lordosis induction and uterine weight in sexually mature**
954 **females**
955 The lordosis quotient was significantly increased by treatment with 17β-estradiol in
956 both Test 1 and 2 (treatment: $F(2,15) = 38.645$, $P < 0.001$; Bonferroni *post hoc* test: P
957 < 0.001, E_2 vs. Oil or TDMPP; Supplemental figure 2). Although there was no
958 statistical difference, five out of six female (83.3%) in TMDPP treated group showed
959 at least one lordosis response whereas only one out of six female (16.7%) in oil
960 treated group showed lordosis response in test 2 (Supplemental table 3). As for
961 uterine weight, both estradiol benzoate and 2,6-TDMPP treatments significantly
962 increased uterine weight ($F(2,15) = 30.331$, $P < 0.001$; Bonferroni *post hoc* test: $P <$
963 0.001, E_2 or TDMPP vs. Oil; Supplemental figure 3), demonstrating that TDMPP
964 shows estrogenic action also in adults, even if the doses necessary for the effect
965 should be higher than in the perinatal period.
966
967 **Reproduction rate in mating**
968 All females of 5 female-male pairs in the control group showed vaginal plugs by the
969 first or second estrous cycle, and 7.8 ± 1.1 pups (mean ± SEM) were delivered from
970 five females. On the other hand, only one out of 5 females in the E_2-low group
971 showed a vaginal plug, and none of them delivered. Similarly, none of the 5 females
972 in the TDMPP-high group showed a vaginal plug, and no delivery occurred. In the
973 TDMPP-low group 3 out of 4 females showed vaginal plugs within 3 estrous cycles,
974 and deliveries occurred in 3 out of 4 females, although all the pups of a pregnant
975 female were dead (Supplemental table 4).
976
977 **Ovarian morphology**

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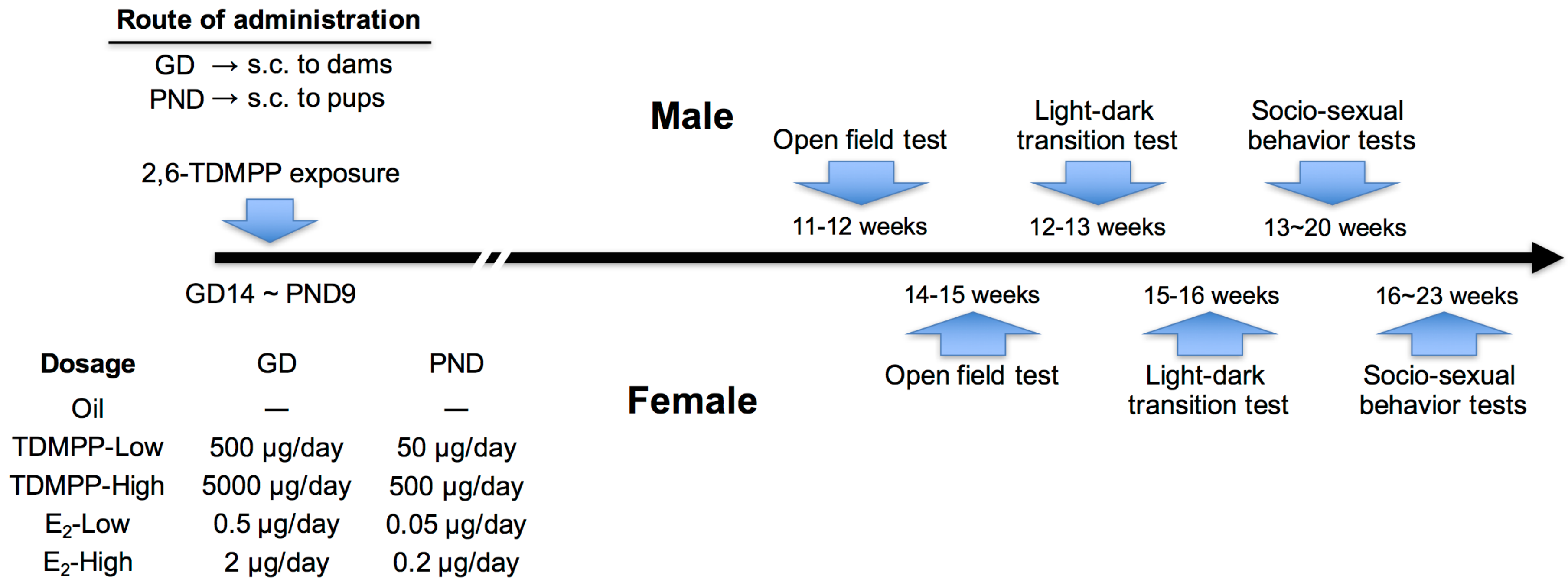
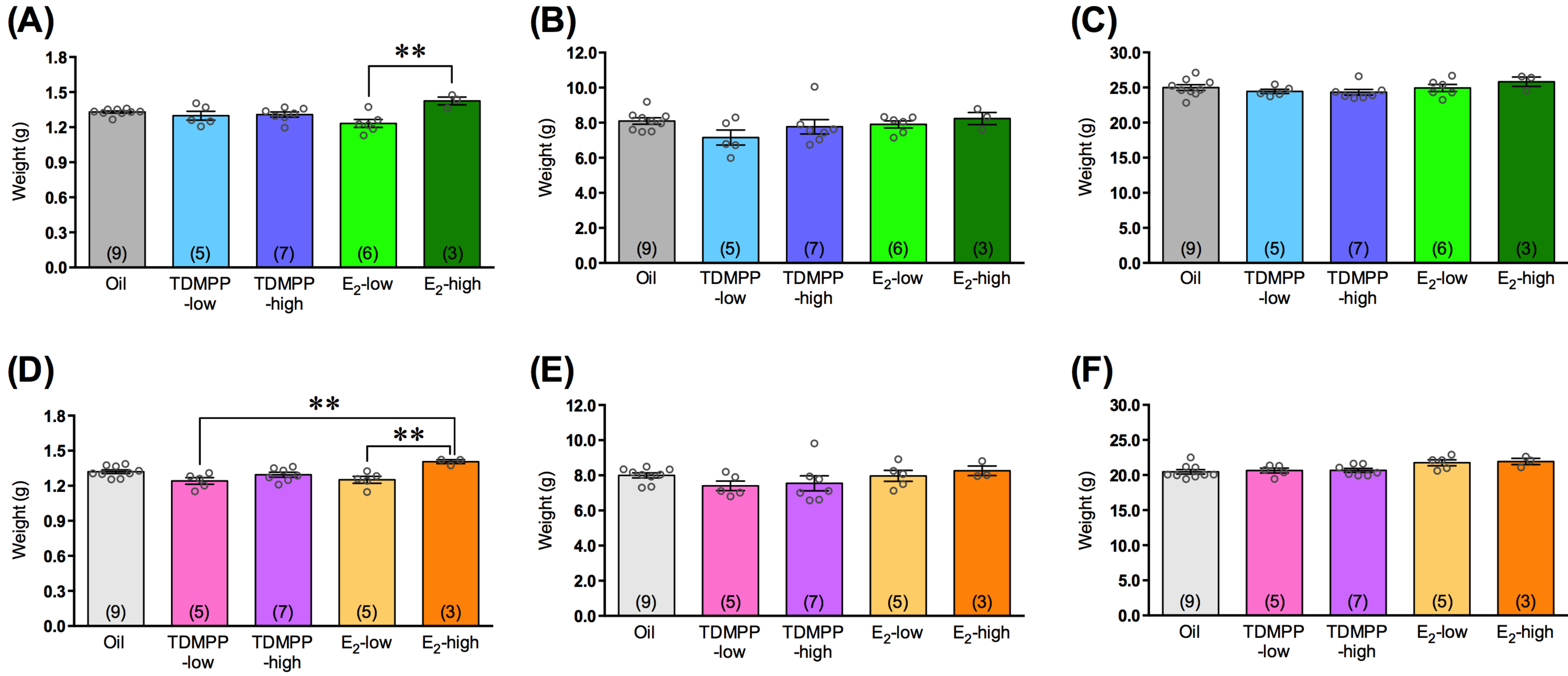
978 In the Oil and TDMPP-low groups, ovarian follicles and the corpus luteum were
979 found. On the other hand, the corpus luteum scarcely appeared in ovaries of
980 TDMPP-high, E_2-low and E_2-high groups, indicating that the release of mature eggs
981 from ovaries was impaired (Supplemental figure 4).
982
983 **Supplemental figure legends**
984
985 **Supplemental Figure 1: The molecular structures of PBDMPP, TDMPP and its 4**
986 **different metabolites.**
987
988 **Supplemental Figure 2: Effect of TDMPP administration on lordosis induction.**
989 The number in parentheses indicate number of animals. The data are presented as the
990 mean ± SEM. ** $P < 0.01$.
991
992 **Supplemental Figure 3: Examination of uterotropic property of TDMPP.** The
993 number in parentheses indicate number of animals. The data are presented as the
994 mean ± SEM. ** $P < 0.01$ vs. Oil.
995
996 **Supplemental Figure 4: Effect of TDMPP administration on ovarian**
997 **morphorogy.** Representative photomicrographs of ovary sections from females of
998 each treatment group. Scale bar indicate 200 µm.
999
1000



Dosage	GD	PND
Oil	—	—
TDMPP-Low	500 μg/day	50 μg/day
TDMPP-High	5000 μg/day	500 μg/day
E_2-Low	0.5 μg/day	0.05 μg/day
E_2-High	2 μg/day	0.2 μg/day

Figure 1.



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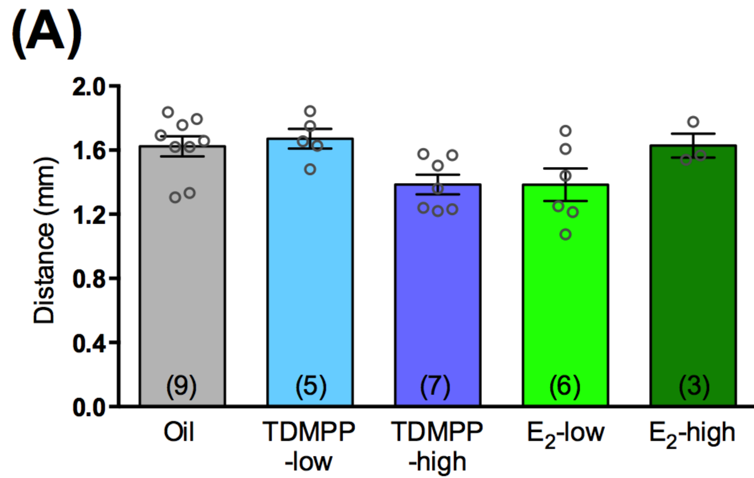

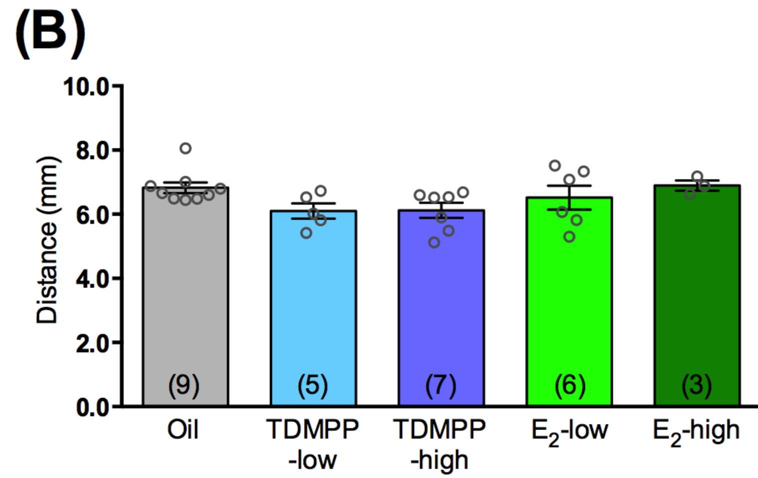

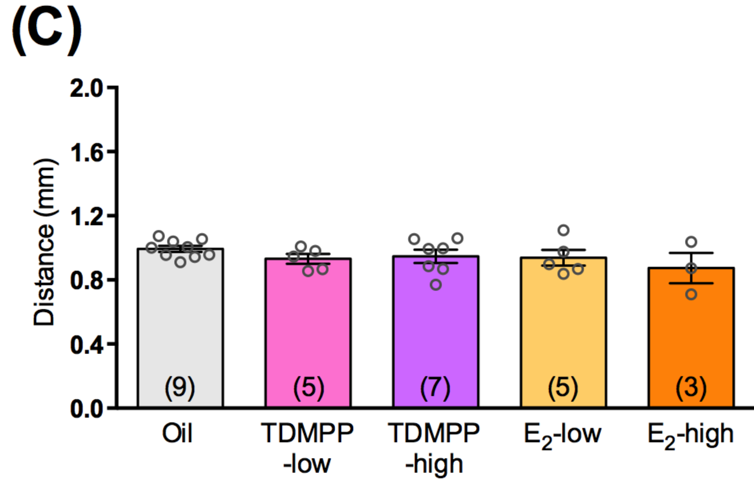

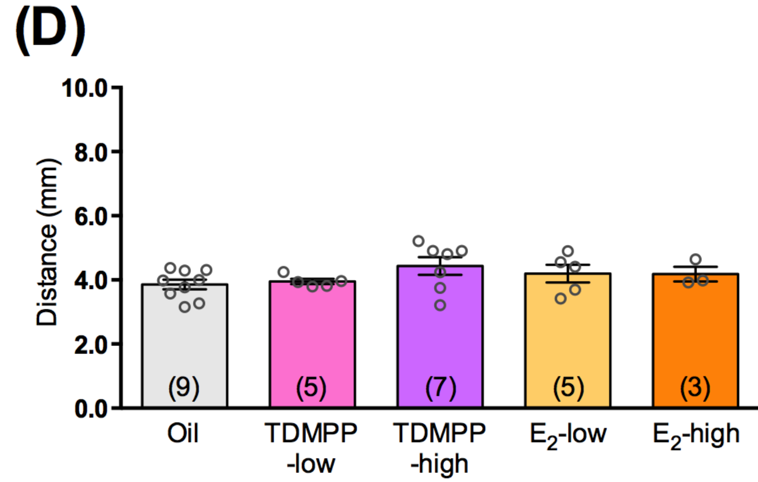

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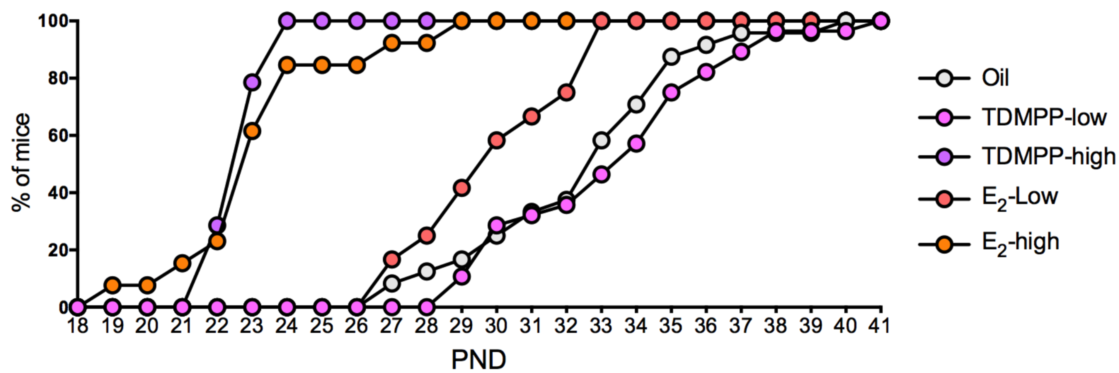

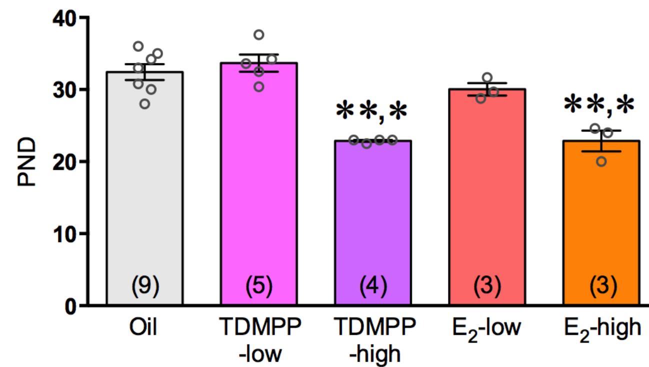

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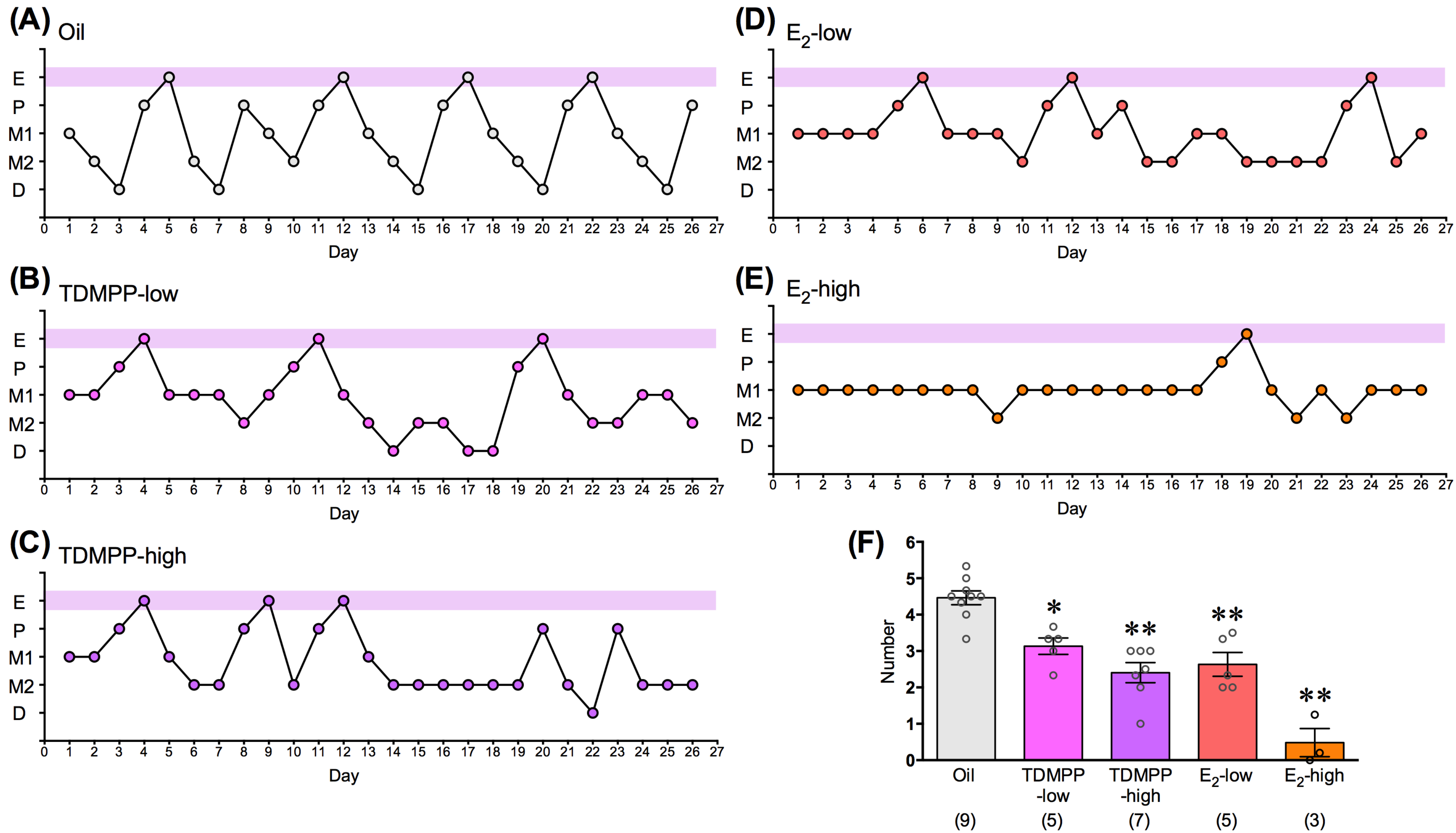

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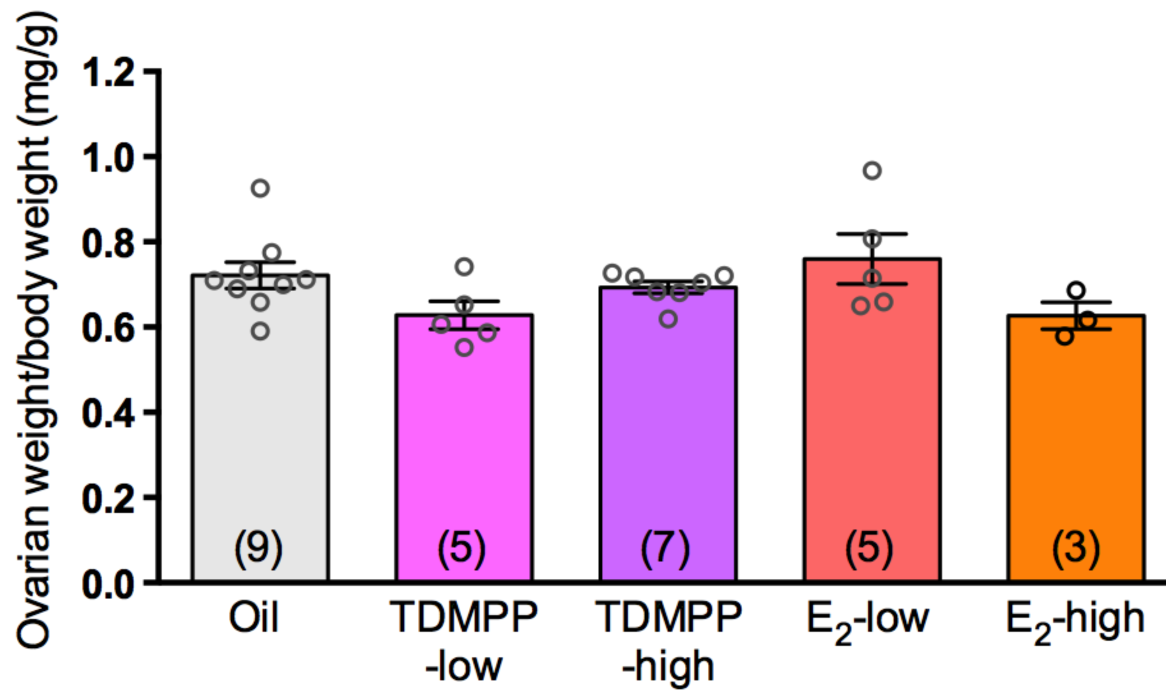

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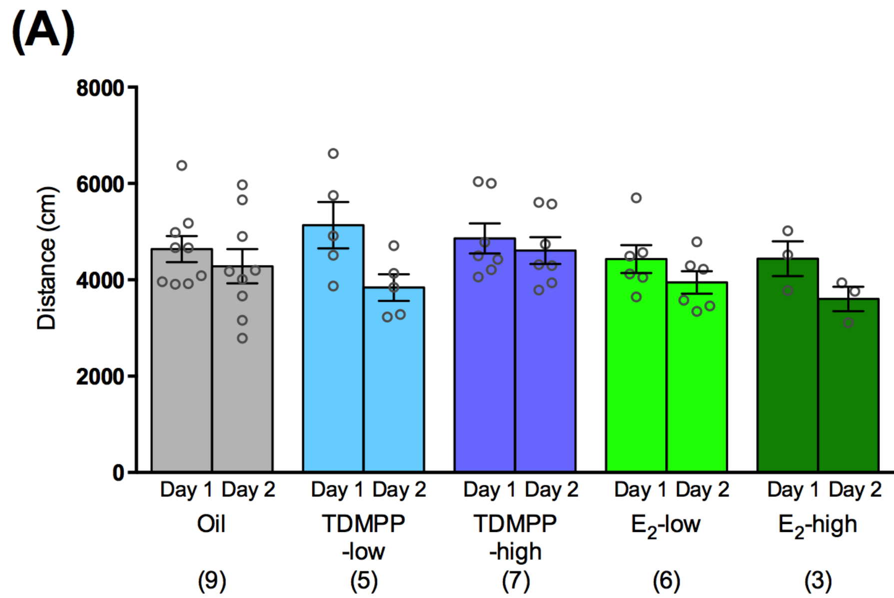

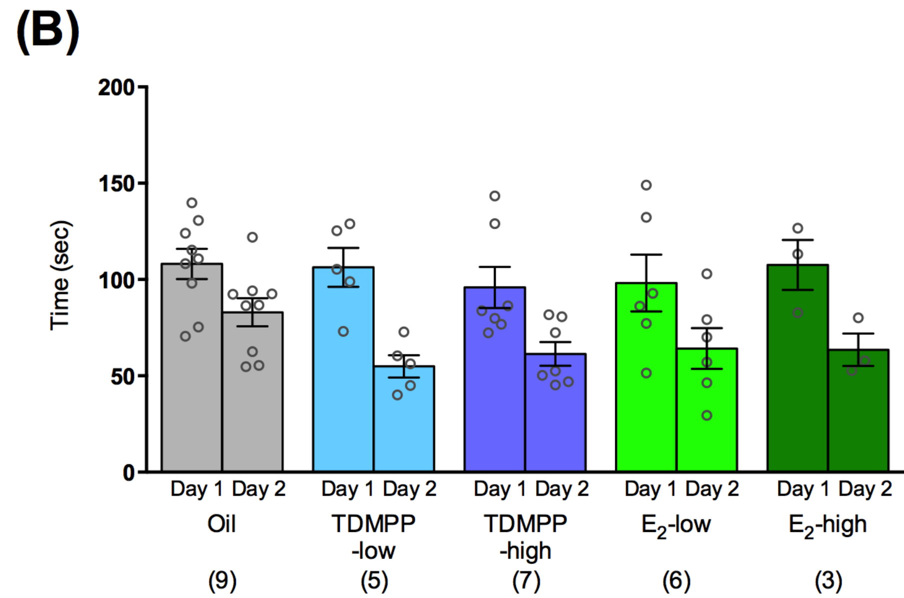

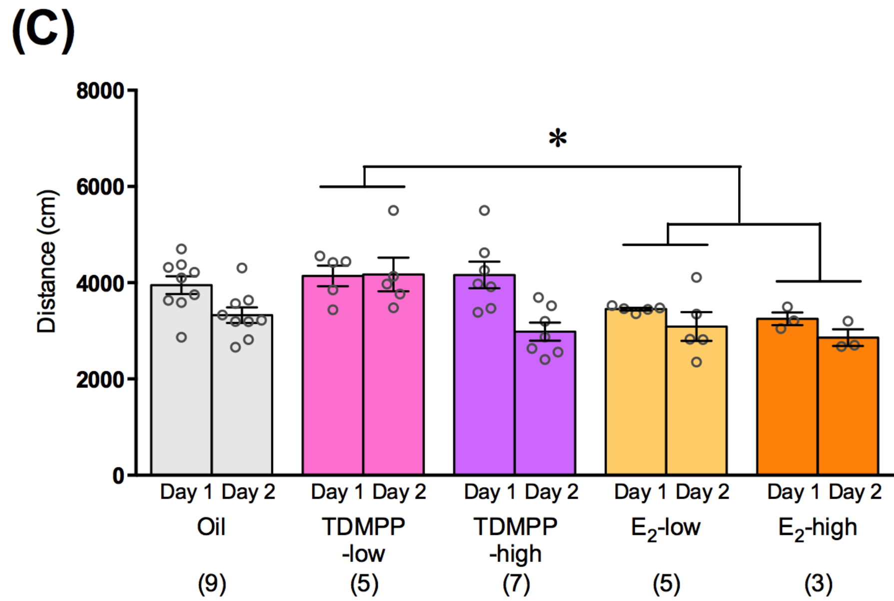

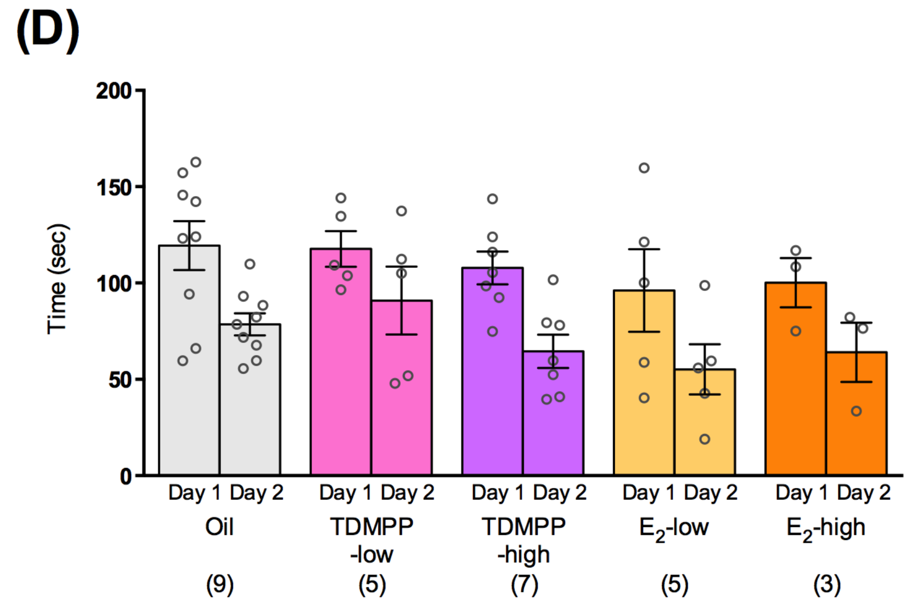

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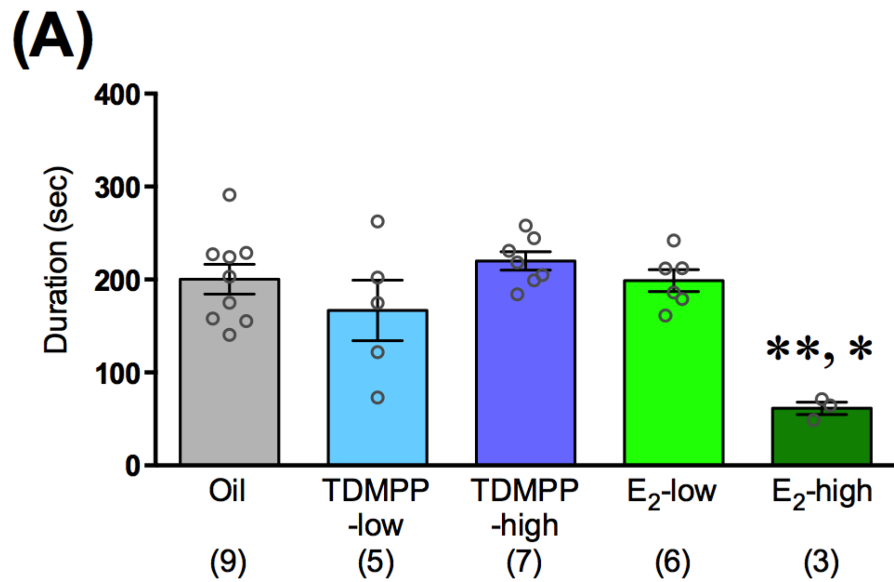

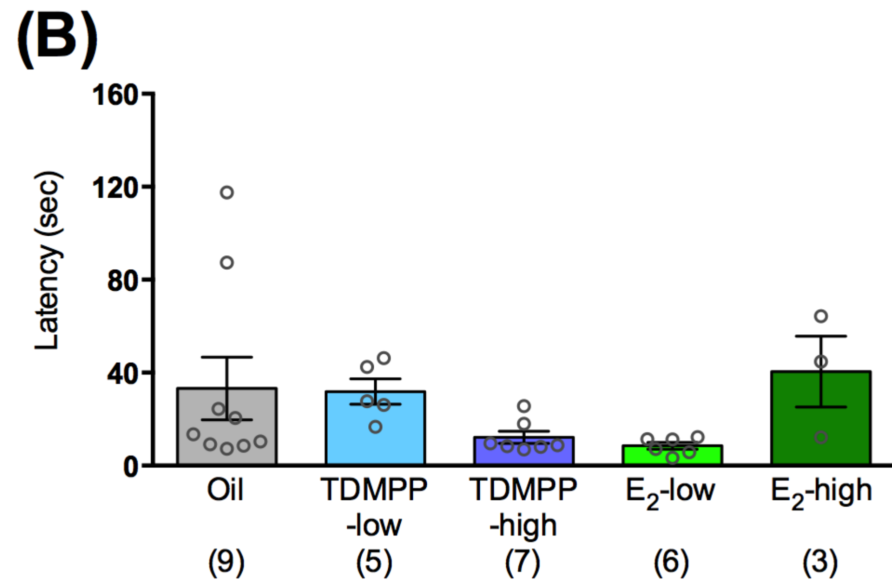

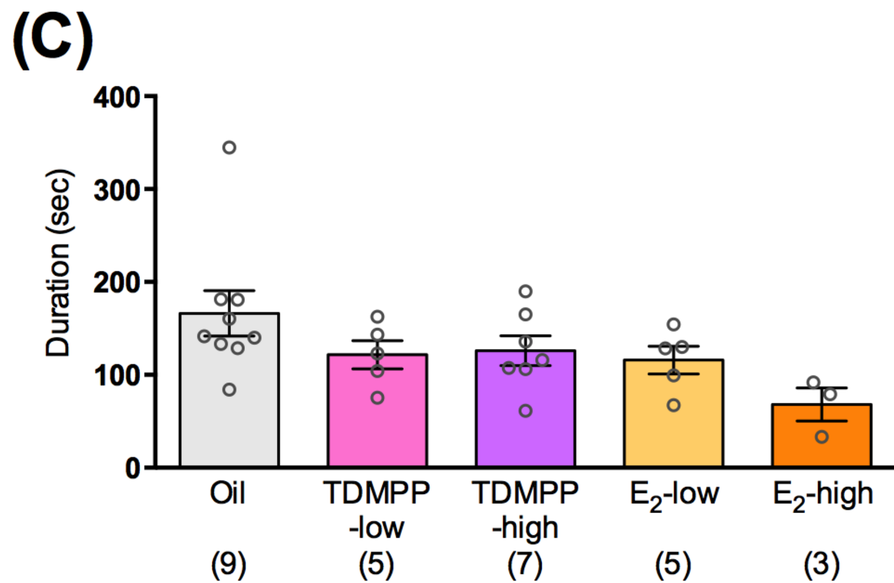

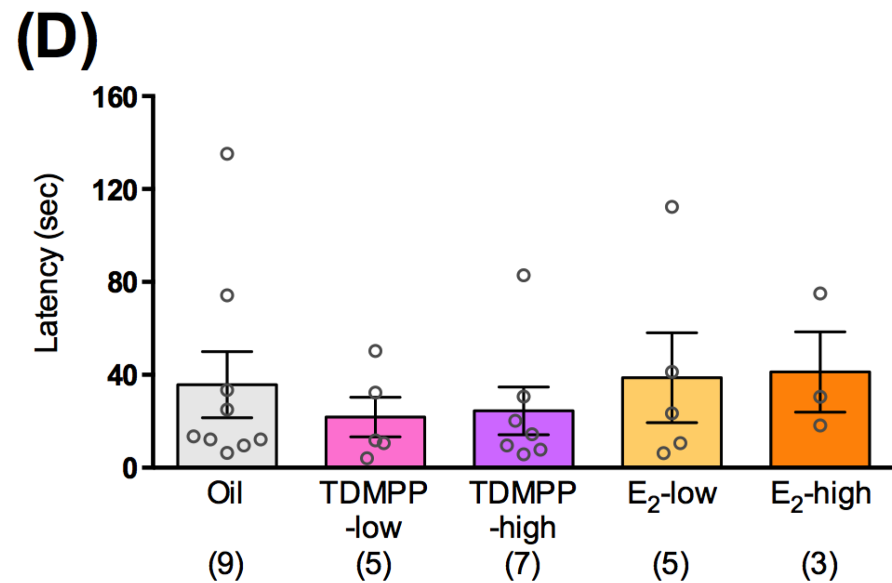

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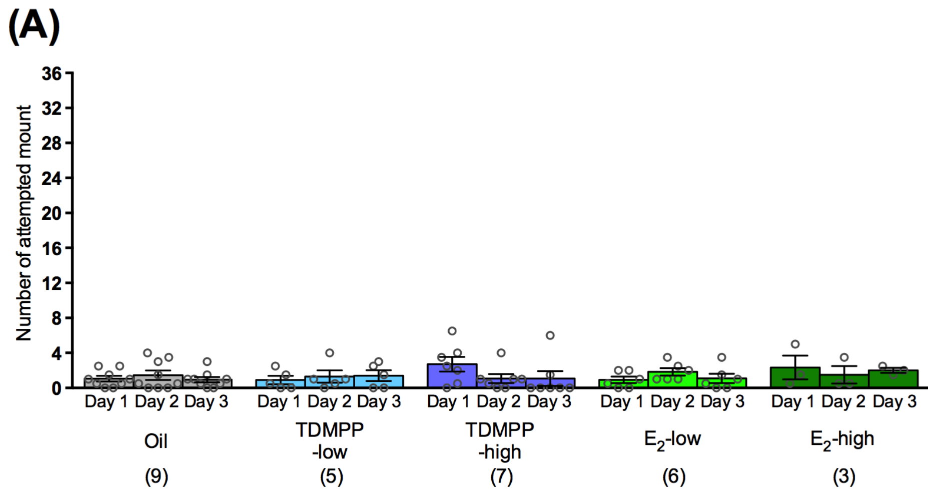

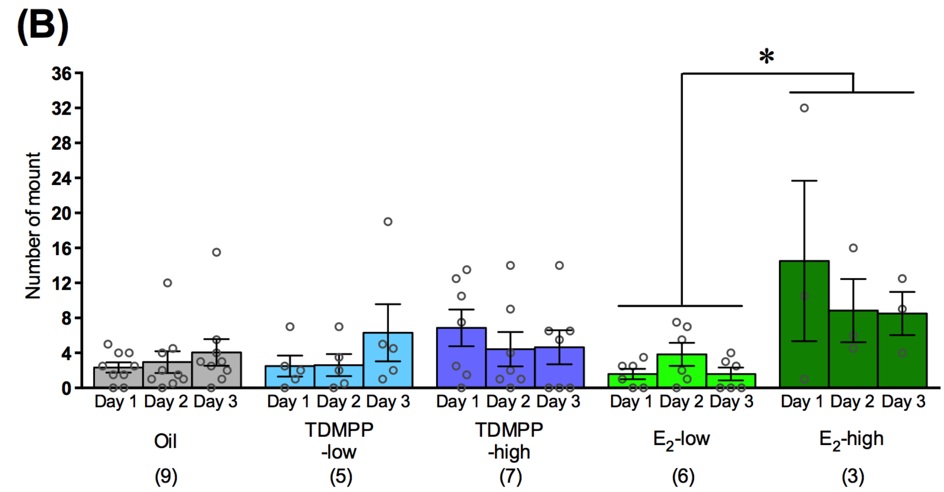

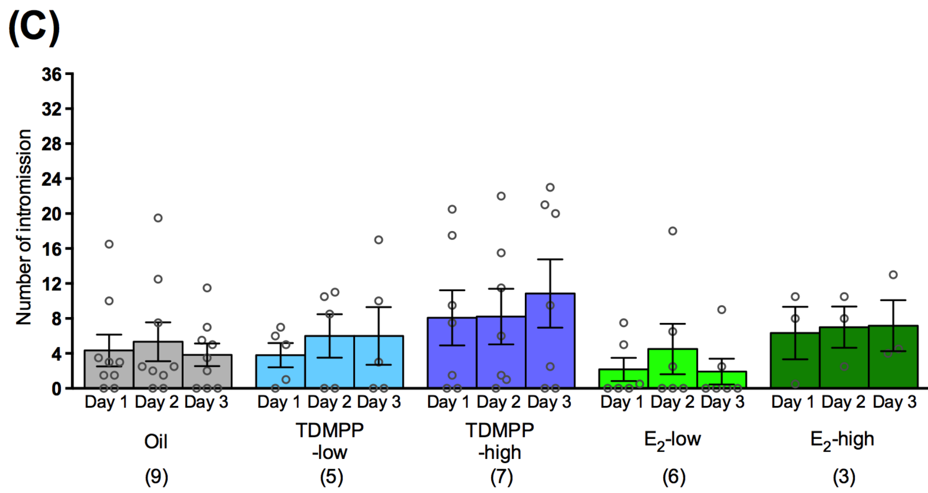

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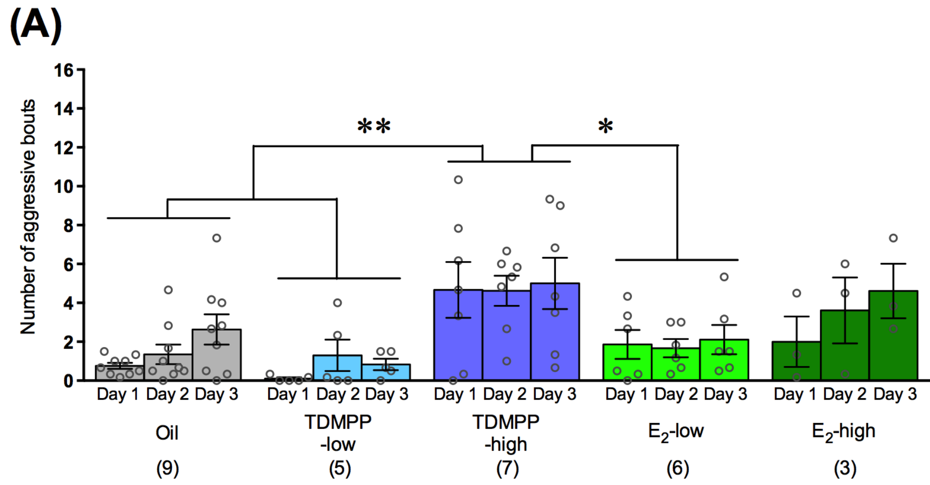

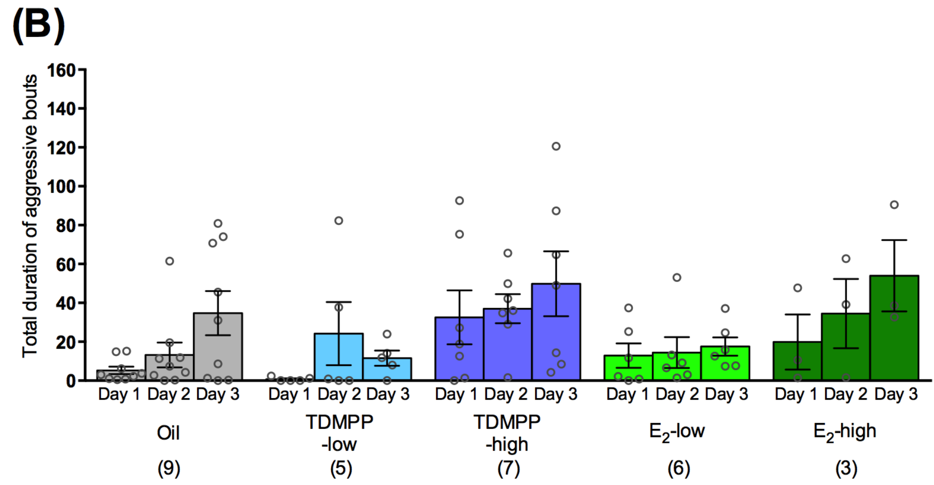

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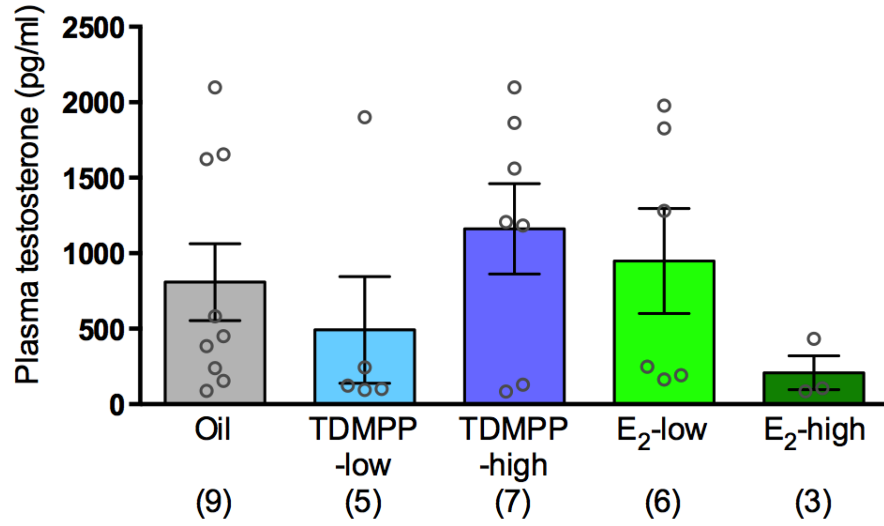

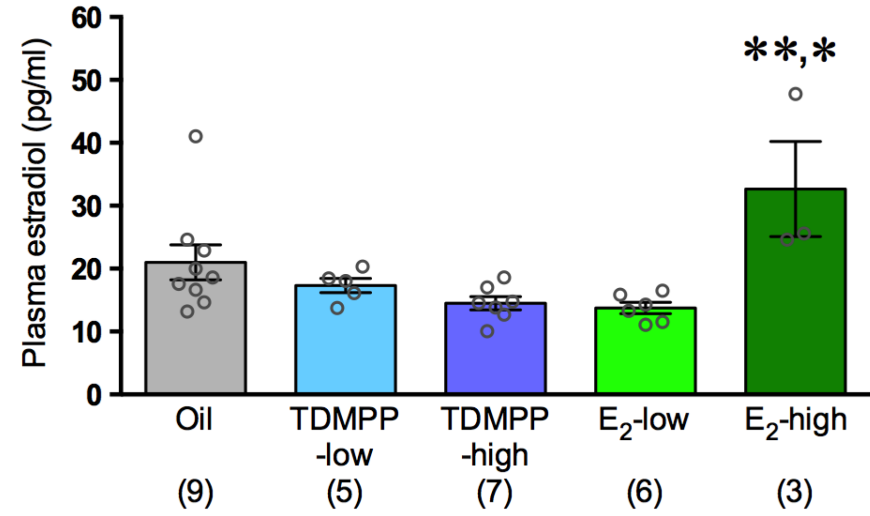

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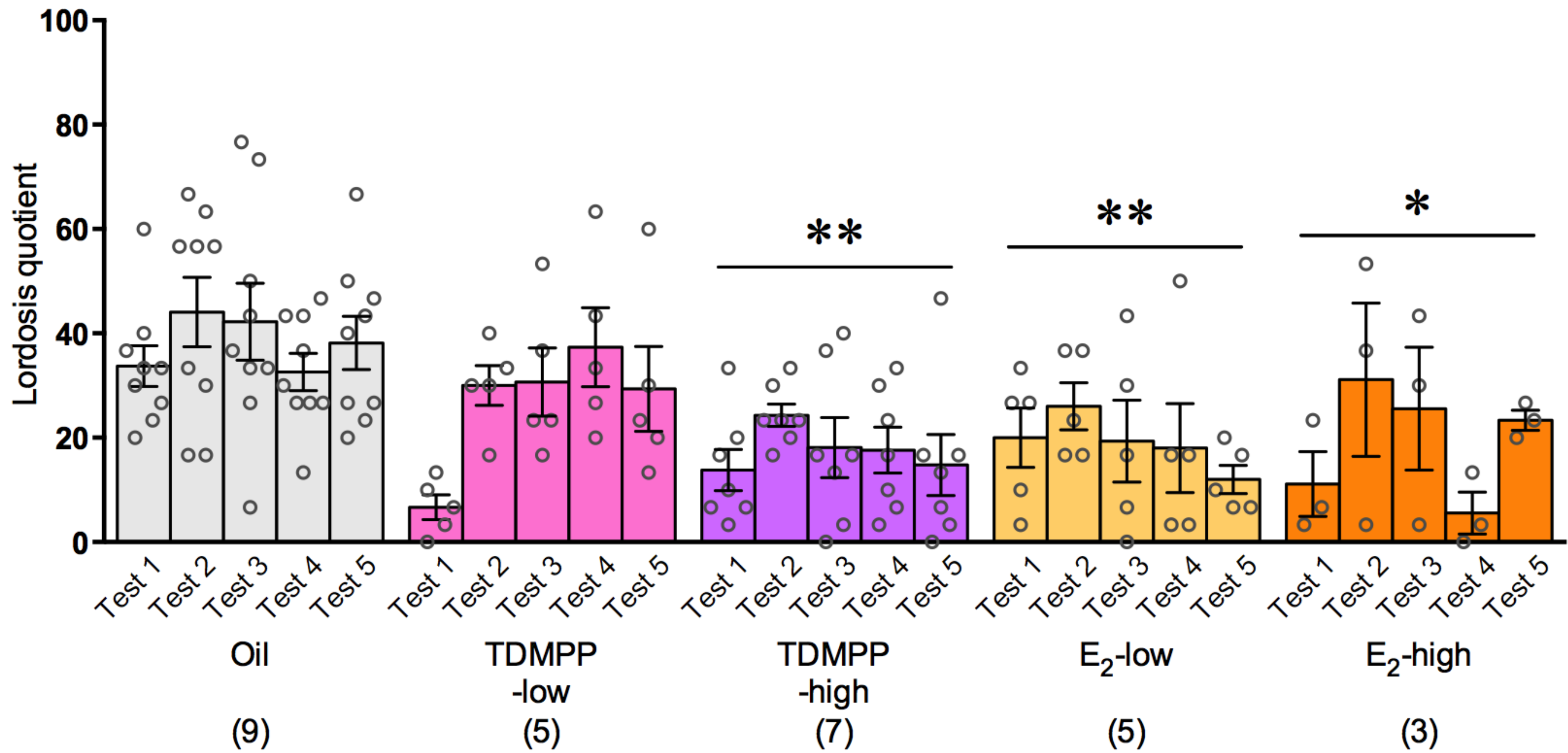

Figure 12.

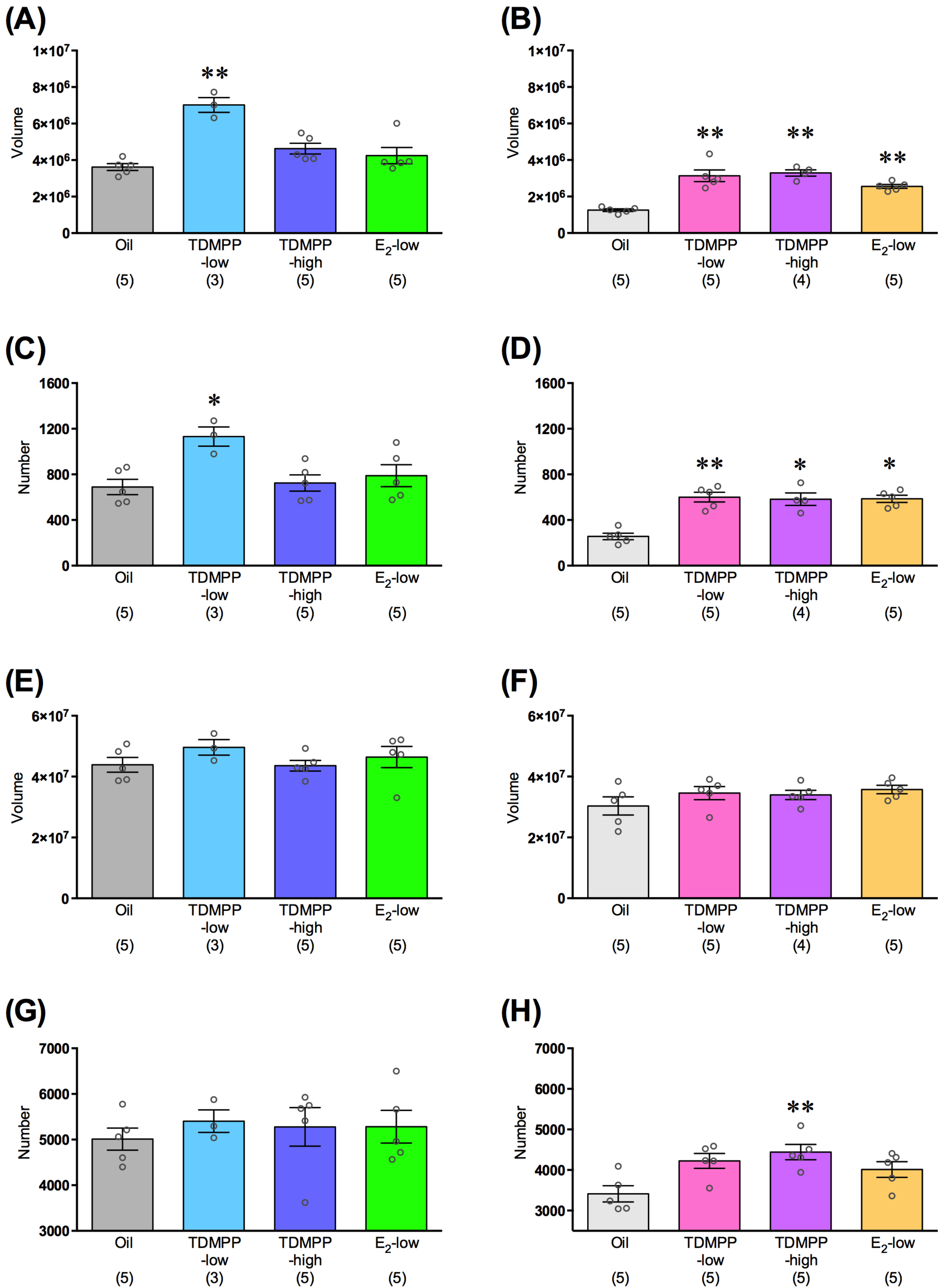

Figure 13.

1 Table 1

2 **Parameters for stereological analyses for Calb-SDN**

3	Oil		TDMPP-low		TDMPP-high		E2	
4								
5	Male	Female	Male	Female	Male	Female	Male	Female
6 No. of sections	9.8±0.67	4.2±0.2	10.3±0.33	7.6±0.51	9±0.32	7.6±0.68	9.2±0.2	7.2±0.37
7 Sampling Grid Size	70×70 µm							
8 Counting frame size	35×35 µm							
9 Number of sampling sites	51.8 ± 4.22	20.4 ± 1.21	98.7 ± 4.91	48 ± 4.16	68.2 ± 3. 85	46.6 ± 4.77	64.6 ± 4.76	41.4 ± 2.98

10 Section thickness, 30 µm, section interval, 30 µm; dissector height 14-16 µm; guard zone height, 1.5 µm

11 **Parameters for stereological analyses for BNSTp**

12	Oil		TDMPP-low		TDMPP-high		E2	
13								
14	Male	Female	Male	Female	Male	Female	Male	Female
15 No. of sections	10.8±0.37	9±0.45	10.7 ± 0.33	9.4 ± 0.4	11.2 ± 0.37	9.6 ± 0.24	10.8±0.2	9.8±0.37
16 Sampling Grid Size	100 × 100 µm							
17 Counting frame size	50 × 50 µm							
18 Number of sampling sites	227 ± 9.9	168 ± 11.53	257.7 ± 9.17	202.7 ± 3.53	226.8 ± 9.30	190 ± 3.65	245 ± 14.24	202.6 ± 7.28

19 Section thickness, 30 µm, section interval, 30 µm; dissector height 14-16 µm; guard zone height, 1.5 µm

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