

Polychlorinated biphenyl (118) activates osteoclasts and induces bone resorption in goldfish

著者	Yachiguchi Koji, Matsumoto Noriko, Haga Yuki, Suzuki Motoharu, Matsumura Chisato, Tsurukawa Masahiro, Okuno Toshihiro, Nakano Takeshi, Kawabe Kimi, Kitamura Kei-ichiro, Toriba Akira, Hayakawa Kazuichi, Chowdhury Vishwajit S., Endo Masato, Chiba Atsuhiko, Sekiguchi Toshio, Nakano Masaki, Tabuchi Yoshiaki, Kondo Takashi, Wada Shigehito, Mishima Hiroyuki, Hattori Atsuhiko, Suzuki Nobuo
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Polychlorinated biphenyl (118) activates osteoclasts and induces bone resorption in goldfish

7 Koji Yachiguchi,¹ Noriko Matsumoto,¹ Yuki Haga,² Motoharu Suzuki,² Chisato Matsumura,² Masahiro Tsurukawa,² Toshihiro Okuno,² Takeshi Nakano,² Kimi Kawabe,³ Kei-ichiro Kitamura,⁴ Akira Toriba,³ Kazuichi Hayakawa,³ Vishwajit S. Chowdhury,⁵ Masato Endo,⁶ Atsuhiko Chiba,⁷ Toshio Sekiguchi,¹ Masaki Nakano,⁸ Yoshiaki Tabuchi,⁹ Takashi Kondo,¹⁰ Shigehito Wada,¹¹ Hiroyuki Mishima¹², Atsuhiko Hattori,⁸ and Nobuo Suzuki^{1*}

14 ¹*Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Housu-gun, Ishikawa 927-0553, Japan*

16 ²*Hyogo Prefectural Institute of Environmental Sciences, Kobe, Hyogo 654-0037, Japan*

18 ³*Faculty of Pharmaceutical Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan*

20 ⁴*Faculty of Health Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kodatsuno, Ishikawa 920-0942, Japan*

22 ⁵*International Education Center, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan*

24 ⁶*Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan*

26 ⁷*Department of Materials and Life Sciences, Sophia University, Tokyo 102-8554, Japan*

28 ⁸*Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba 272-0827, Japan*

30 ⁹*Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Sugitani, Toyama 930-0194, Japan*

32 ¹⁰*Department of Radiological Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani, Toyama 930-0194, Japan*

35 ¹¹*Department of Oral and Maxillofacial Surgery, Faculty of Medicine, University of Toyama, Sugitani, Toyama 930-0194, Japan*

37 ¹²*Kochi Gakuen College, Kochi 780-0955, Japan*

39 Running title: PCB promotes bone resorption in fish

40 *Correspondence to: Dr. N. Suzuki, Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Ogi, Noto-cho, Ishikawa 927-0553, Japan. Tel.: 81-768-74-1151; Fax 81-768-74-1644;

43 e-mail: nobuos@staff.kanazawa-u.ac.jp

44 **Abstract**

45 *Purpose:* To analyze the effect of Polychlorinated biphenyl (PCB118) on fish bone
46 metabolism, we examined osteoclastic and osteoblastic activities, as well as
47 plasma calcium levels, in the scales of PCB (118)-injected goldfish. In addition,
48 effect of PCB (118) on osteoclasts and osteoblasts was investigated *in vitro*.

49 *Methods:* Immature goldfish, in which the endogenous effects of sex steroids are
50 negligible, were used. PCB (118) was solubilized in dimethyl sulfoxide at a
51 concentration of 10 ppm. At 1 and 2 days after PCB (118) injection (100 ng /g
52 body weight), both osteoclastic and osteoblastic activities, and plasma calcium
53 levels were measured. In an *in vitro* study, then, both osteoclastic and osteoblastic
54 activities as well as each marker mRNA expression were examined.

55 *Results:* At 2 days, scale osteoclastic activity in PCB (118)-injected goldfish
56 increased significantly, while osteoblastic activity did not change significantly.
57 Corresponding to osteoclastic activity, plasma calcium levels increased
58 significantly at 2 days after PCB (118) administration. Osteoclastic activation was
59 also occurred in the marker enzyme activities and mRNA expressions *in vitro*.
60 Thus, we conclude that PCB (118) disrupts bone metabolism in goldfish both *in*
61 *vivo* and *in vitro* experiments.

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63 **Keywords:** PCB (118), bone metabolism, fish scales, osteoclasts, osteoblasts,
64 plasma calcium

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78 **1. Introduction**

79 It has been reported that polychlorinated biphenyl (PCB) congeners act as
80 endocrine-disrupting compounds (Lind et al. 2004; Bovee et al. 2011; Nakayama
81 et al. 2011; Ju et al. 2012). As bone formation and resorption are controlled by
82 several hormones and vitamins (see a review, Peacock 2010), PCBs might disturb
83 bone metabolism. In some animals, actually, the bone disruption caused by PCB
84 has been reported (rat: Lind et al. 2004a; bear: Sonne et al. 2004; sheep: Gutleb et
85 al. 2010; alligator: Lind et al. 2004b; turtle: Holliday DK and Holliday CM 2012;
86 salmon: Olufsen and Arukwe 2011; zebrafish: Ju et al. 2012). In humans, changes
87 in bone metabolism associated with exposure to PCBs have also been investigated
88 (Hodgson et al. 2008). However, the direct effects of PCBs on osteoclasts and
89 osteoblasts have not yet been elucidated in any animals.

90 The teleost scale is a calcified tissue that contains osteoblasts, osteoclasts, and
91 the bone matrix of two layers (bony layer: a thin, well-calcified external layer; a
92 fibrillary layer: a thick, partially calcified layer) (Bereiter-Hahn and Zylberberg
93 1993; Suzuki et al. 2000; Yoshikubo et al. 2005; Suzuki et al. 2007; Ohira et al.
94 2007). The bone matrix, which includes type I collagen (Zylberberg et al. 1992),
95 osteocalcin (Nishimoto et al., 1992), and hydroxyapatite (Onozato and Watabe
96 1979) is present in the scale as well as in mammalian bone. Recently, we detected
97 both cathepsin K and tartrate-resistant acid phosphatase (TRAP) mRNA
98 expression in scale osteoclasts (Azuma et al. 2007). In osteoblasts, we detected
99 osteoblast-specific markers, such as alkaline phosphatase (ALP), runt-related
100 transcription factor 2, osterix, osteocalcin, type I collagen, and the receptor
101 activator of the NF- κ B ligand (Thamamongood et al. 2012). Therefore, the
102 features of osteoclasts and osteoblasts in scales are similar to those in mammals.

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103 In fish as well as mammals, plasma calcium level was regulated by hormones
104 such as parathyroid hormone (Suzuki et al. 2011a) and calcitonin (Suzuki et al.
105 2000; Suzuki et al. 2004a). In an *in vivo* experiment, fugu parathyroid hormone I
106 induced hypercalcemia resulted from the increase of both osteoblastic and
107 osteoclastic activities in the scale and caused to decrease scale calcium contents
108 (Suzuki et al. 2011a). Scale osteoclastic activation was also observed in the
109 prostaglandin E₂ injected-goldfish (Omori et al. 2012). It is reported that the
110 scales are a better potential internal calcium reservoir than the body skeletons,
111 jaws and otolithes, examined by the ⁴⁵Ca-labelling study for the calcified tissues
112 of goldfish and killifish (Mugiya and Watabe 1977). Thus, we conclude that
113 teleost scale is an active and functional calcium reservoir.

114 In fish, PCB (118) is the highest congeners compared with PCB-105, -156,
115 -167, -123, -157, -114, -189, -77, -126, -81, or -169 (Bhavsar et al. 2007).
116 Furthermore, it has been reported that trabecular bone mineral content was almost
117 30% lower in the PCB (118) (49 µg/kg body wt/day) at the metaphysis in sheep
118 (Gutleb et al. 2010), although the detail mechanism has not yet been elucidated.
119 We therefore analyzed the effect of PCB (118) on scale osteoclastic and
120 osteoblastic activities, as well as plasma calcium levels, in the goldfish scales. In
121 addition, effect of PCB (118) on osteoclasts and osteoblasts was investigated *in*
122 *vitro*. This is the first to demonstrate that PCB (118) activates osteoclasts and
123 induced bone resorption in fish.

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125 2. Materials and methods

126 *Animals*

127 To examine the effect of PCB (118) on the bone metabolism, immature
128 goldfish (4-6 g), in which the endogenous effects of sex steroids are negligible,

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129 were used for the *in vivo* study. A previous study (Suzuki et al. 2000) indicated
130 that the sensitivity for calcemic hormones was higher in mature female than in
131 mature male teleosts. Therefore, female goldfish (*Carassius auratus*) (30 - 40 g)
132 were purchased from commercial source (Higashikawa Fish Farm,
133 Yamatokoriyama, Japan) and used for the *in vitro* experiments.

134 All experimental procedures were conducted in accordance with the Guide
135 for the Care and Use of Laboratory Animals prepared by Kanazawa University.

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137 *Effects of PCB (118) on scale osteoclastic and osteoblastic activities and the*
138 *plasma calcium in goldfish at day-1 and -2 after PCB (118) injection (in vivo*
139 *experiment)*

140 PCB (118) was solubilized in dimethyl sulfoxide (DMSO) at a concentration
141 of 10 ppm. Goldfish (body weight: 4 - 6 g) were anesthetized with ethyl 3-
142 aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA)
143 and taken the blood (about 100 μ l) from caudal vessels of each individual into
144 heparinized syringes just before PCB (118) injection. After centrifugation at
145 15,000 rpm for 3 min, the plasma was immediately frozen and kept at -80 °C until
146 use. In the experimental group (n = 10), thereafter, PCB (118) was
147 intraperitoneally injected (100 ng/g body weight). The goldfish in the control
148 group (n = 10) were injected with DMSO in the same manner. These goldfish
149 were kept in the aquarium for 1 and 2 days. During the experimental periods,
150 these goldfish were not given any food to exclude intestinal calcium uptake from
151 diets. Each day after injection, the scales were collected from each goldfish. At
152 day-2 after injection, blood samples (about 100 μ l) were collected from the gill
153 using a heparinized capillary from individual, anesthetized goldfish. After
154 centrifugation at 15,000 rpm for 3 min, the plasma was also immediately frozen

155 and kept at -80°C until use. The plasma total calcium level (mg/100 ml) was
156 determined using an assay kit (Calcium C, Wako Pure Chemical Industries, Ltd.,
157 Osaka, Japan). Then, we measured the activities of ALP and TRAP activities as
158 respective indicators of each activity in osteoclasts and osteoblasts (Suzuki et al.
159 2000; Suzuki et al. 2002; Suzuki et al. 2009). The measurement methods (Suzuki
160 et al. 2009) of ALP and TRAP activities were as follows. The incubated scale was
161 transferred to its own well in a 96-well microplate after washing with saline. An
162 aliquot of 100 μl of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM MgCl_2 ;
163 0.1 mM ZnCl_2) for ALP activity or an acid buffer (0.1 M sodium acetate including
164 20 mM tartrate, pH 5.3) for TRAP activity was added to each well. This
165 microplate was frozen at -80°C immediately and then kept at -20°C until analysis.
166 After thawing, an aliquot of 100 μl of 20 mM para-nitrophenyl-phosphate in an
167 alkaline buffer or an acid buffer was added to each well. This plate was then
168 incubated at 20°C for 30 min with shaking. After incubation, the reaction was
169 stopped by adding 50 μl of a 3 N NaOH-20 mM EDTA solution. Aliquots of 150
170 μl of a colored solution were transferred to a new plate, and the absorbance was
171 measured at 405 nm. The absorbance was converted into the amount of produced
172 para-nitrophenol (pNP) using a standard curve for pNP. After measurement of the
173 absorbance, the ALP and TRAP activities were normalized by the surface area
174 (mm^2) of each goldfish scale. The results are shown as the means \pm SE of eight
175 scales.

176 177 *PCB (118) contents in the scales of goldfish (in vivo experiment)*

178 At day-1 and -2 after PCB (118) injection, the scales were collected from
179 goldfish and then immediately frozen and kept at -80°C until use. The PCB (118)
180 contents were analyzed by the methods of Hirai et al. (2005). Because a single

181 sample volume was very small, we conducted three measurements to obtain a
182 pulled sample. Thus, the mean of three measurements was described in the results.
183
184 *Effects of PCB (118) on osteoclastic and osteoblastic activities in the cultured*
185 *scales of goldfish (in vitro experiment)*
186 Scales collected from goldfish (n = 10) after anesthesia with ethyl 3-
187 aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich) and incubated for 6
188 and 18 h in Leibovitz's L-15 medium (Invitrogen, Grand Island, NY, USA)
189 containing a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., OH,
190 USA) supplemented with PCB (118) (0.025, 0.25, and 2.5 ppm). In an *in vivo*
191 experiment, around 0.1 to 0.05 ppm PCB was detected in the PCB-injected scales.
192 Based on these PCB contents in the scales, we decided the administration doses of
193 PCB in an *in vitro* experiment. The PCB concentration in one goldfish was
194 performed using 48 scales from each left or right side. The 48 scales used in the
195 present study were considered to use as follows: 1) 8 scales for TRAP analysis by
196 0.025 ppm, 2) 8 scales for TRAP analysis by 0.25ppm, 3) 8 scales for TRAP
197 analysis by 2.5 ppm, 4) 8 scales for ALP analysis by 0.025 ppm, 5) 8 scales for
198 ALP analysis by 0.25ppm, 6) 8 scales for ALP analysis by 2.5 ppm. The
199 respective mean for TRAP (obtained from 8 individual scales of one goldfish) and
200 ALP (obtained from 8 individual scales of one goldfish) activities from left side
201 (experimental group) was compared with those of right side (control group).
202 Using 10 individual goldfish, same experiment was done repeatedly. The
203 experiments for 0.25 and 2.5 ppm PCB (118) were carried out in the same
204 manner. After incubation, TRAP and ALP activities were measured using the
205 same methods described above (Suzuki et al. 2009). The results are shown as
206 means \pm SEM (n = 10).

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208 *Changes in TRAP, cathepsin K and RANKL mRNA expressions in PCB (118)-*
209 *treated goldfish scales (in vitro experiment)*

210 Scales were collected from goldfish under anesthesia with ethyl 3-
211 aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich). To examine
212 changes in TRAP, cathepsin K, and RANKL mRNAs that responded to PCB
213 (118), these scales were incubated for 18 h in Leibovitz's L-15 medium
214 (Invitrogen) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals).
215 In the prostaglandin E₂-treated scales of goldfish, we previously reported that
216 TRAP, cathepsin K, RANKL mRNA expression increased at 18 h of incubation
217 (Omori et al. 2012). Therefore, this incubation period was adopted. After
218 incubation, the scales were frozen at -80 °C for mRNA analysis.

219 Total RNAs were prepared from goldfish scales using a total RNA isolation
220 kit for fibrous tissue (Qiagen GmbH, Hilden, Germany). Complementary DNA
221 synthesis was performed using a kit (Qiagen GmbH). Gene-specific primers for
222 TRAP (sense: 5'-AACTTCCGCATTCCTCGAACAG-3'; antisense: 5'-
223 GGCCAGCCACCAGGAGATAA-3') (Azuma et al. 2007), cathepsin K (sense:
224 5'-GCTATGGAGCCACACCAAAGG-3'; antisense: 5'-
225 CTGCGCTTCCAGCTCTCACAT-3') (Azuma et al. 2007), and RANKL (sense:
226 5'-GCGCTTACCTGCGGAATCATATC-3'; antisense: 5'-
227 AAGTGCAACAGAATCGCCACAC-3') (Suzuki et al. 2011a) were used. The
228 amplification of β -actin cDNA using a primer set (5':
229 CGAGCGTGGCTACAGCTTCA; 3': GCCCGTCAGGGAGCTCATAG) (Azuma
230 et al. 2007) was performed. The PCR amplification was analyzed by real-time
231 PCR apparatus (Mx3000p, Agilent Technologies, CA, USA) (Suzuki et al.
232 2011a). The annealing temperature of TRAP, cathepsin K, RANKL, and β -actin

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233 was 60 °C. The TRAP, cathepsin K and RANKL mRNA levels were normalized
234 to the β -actin mRNA level.

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236 *Statistical analysis*

237 All results are expressed as the means \pm SE (n = 10). The statistical
238 significance between control and experimental group was assessed by Student's *t*-
239 test (*in vivo* experiment) or paired *t*-test (*in vitro* experiment). In all cases, the
240 selected significance level was $P < 0.05$.

241

242 **3. Results**

243 *Effects of PCB (118) on scale osteoclastic and osteoblastic activities and the* 244 *plasma calcium in goldfish at 1 and 2 days after PCB (118) injection in vivo*

245 We measured the activities of ALP and TRAP activities as respective
246 indicators of each activity in osteoclasts and osteoblasts. At day-2, scale TRAP
247 activity in PCB-injected goldfish increased significantly (Fig. 1a), while ALP
248 activity did not change significantly at day-1 and -2 (Fig. 1b).

249 Corresponding to the elevation of osteoclastic activity, plasma calcium levels
250 increased significantly at day-2 after PCB administration (Fig. 2)

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252 *PCB (118) contents in the scales of goldfish in vivo*

253 At day-1 and -2 after PCB (118) injection, PCB (118) was detected in the
254 scales. At day-1, PCB contents in the control and PCB-injected scales were
255 determined as 0.39 and 79 (ng/g-wet), respectively. At day-2, PCB (ng/g-wet) of
256 0.38 and 55 was detected in the control and PCB-injected scales, respectively.

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258 *Effect of PCB (118) on osteoclastic and osteoblastic activities in the cultured*
259 *scales of goldfish in vitro*

260 PCB (118) significantly increased the TRAP activities of the scales by 6 h of
261 incubation ($P < 0.05$ for 0.25 ppm) (Fig. 3a). At 18 h of incubation, the TRAP
262 activities in the PCB (118)-treated scales also significantly increased ($P < 0.05$ for
263 0.025 and 2.5 ppm; $P < 0.001$ for 0.25 ppm) (Fig. 4a).

264 In case of the ALP activities, it significantly increased ($P < 0.05$) only by the
265 concentration of 2.5 ppm at the 6 and 18 h incubation (Figs. 3b and 4b).

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267 *Changes in TRAP, cathepsin K and RANKL mRNA expressions in PCB (118)-*
268 *treated goldfish scales in vitro*

269 The mRNA expression of osteoclastic markers (TRAP and cathepsin K)
270 increased significantly by PCB (118) (0.25 ppm) treatment (Figs. 5a and 5b).

271 Similar results were obtained in RANKL. The mRNA expression of RANKL,
272 an activating factor of osteoclasts, increased significantly in the osteoblasts in the
273 PCB (118)-treated scales (Fig. 5c).

274

275 **4. Discussion**

276 In the present study, we are the first to demonstrate that PCB (118) induced
277 hypercalcemia resulting from increasing osteoclastic activity *in vivo*. In an *in vitro*
278 experiment, the data were reproduced and osteoclastic marker mRNA expression
279 as well as enzyme activity increased. In fish, PCB (118) is the highest congeners
280 compared with PCB-105, -156, -167, -123, -157, -114, -189, -77, -126, -81, or -
281 169 (Bhavsar et al. 2007). In aquatic environment, PCB (118) was detected (Hope
282 2008; Aksoy et al. 2011). Therefore, we paid attention to bone metabolism by
283 PCB (118) pollution.

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284 At day-1 and -2 after PCB (118) injection intraperitoneally, we detect PCB
285 (118) in the scale. As described in the Introduction, the scales are potential
286 internal calcium reservoir than the body skeletons, jaws and otolithes. Lake et al.
287 (2006) reported that the correlation between the total mercury concentration of the
288 scales and that of the muscles was high ($r = 0.89$). In sheep, PCB was
289 accumulated and detected in bone at 2 months after administration (Jan et al.
290 2006). We therefore suggest that scale PCB content can be used as an
291 environmental PCB monitor to estimate the environmental pollution of PCB.

292 In the present study, we measured hydroxy-PCB which is a kind of
293 metabolites from PCB because hydroxy-PCB possessed specific and competitive
294 interactions with the plasma thyroid hormone transport protein, transthyretin
295 (Lans et al. 1993). In PCB-treated scales, however, hydroxyl-PCB was not
296 detected. Therefore, this phenomenon of osteogenesis seems to be direct action of
297 PCB (118).

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298 In an *in vivo* experiments, osteoblastic activity increased by the high
299 concentration of PCB (118)(2.5ppm). This indicates that PCB (118) is affected on
300 osteoblasts. Osteogenesis is regulated by osteoblasts (Suda et al. 1999; Teitelbaum
301 2000; Lacey et al. 2012). RANKL produced by cells in the osteoblast lineage
302 binds to the receptor activator of NF- κ B (RANK) in mononuclear hemopoietic
303 precursors and promotes the formation and activity of multinucleated osteoclasts
304 (Suda et al. 1999; Teitelbaum 2000; Lacey et al. 2012). Our present study
305 indicated that RANKL mRNA expression was promoted by PCB (118) treatment.
306 In addition, osteoclastic marker (TRAP and cathepsin K) mRNA expression also
307 increased significantly. Therefore, we strongly suggest that PCB (118) promotes
308 osteoclastogenesis by the RANK-RANKL pathway.

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309 In the present study, we succeeded to analysis the PCB (118) on osteoclasts
310 and osteoblasts. Our results suggest that scale is a good model for analysis of bone
311 metabolism. We previously demonstrated that the osteogenesis of regenerating
312 scale is very similar to that of mammalian membrane bone and a good model of
313 osteogenesis (Yoshikubo et al. 2005). Using this system, furthermore, we first
314 demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic
315 activity in teleosts as well as in mammals (Suzuki et al. 2000) and that melatonin,
316 a major hormone secreted from the pineal gland, suppressed the functions in both
317 osteoclasts and osteoblasts (Suzuki and Hattori 2002). Osteoblasts in the scale
318 responded to estrogen as they do in mammalian bone (Yoshikubo et al. 2005). In
319 addition, the effects of endocrine disrupters, such as bisphenol-A (Suzuki and
320 Hattori 2003) and tributyltin (Suzuki et al. 2006), and heavy metals, i.e., cadmium
321 and mercury (Suzuki et al. 2004b; Suzuki et al. 2011b), on osteoblasts and
322 osteoclasts have been examined. Moreover, we indicated that cadmium (even at
323 10^{-13} M) responded to TRAP activity in the scale (Suzuki et al. 2004b).

324 In conclusion, PCB (118) disrupts bone metabolism in goldfish both *in vivo*
325 and *in vitro* experiments. Our results suggest that PCB (118) promotes
326 osteoclastogenesis by the RANK-RANKL pathway. Furthermore, our previous
327 and present results indicate that scale assay system will be useful for analysis of
328 environmental contaminant on bone metabolism and findings of PCB (118) on
329 bone in fish may be tied in to an overall health issue for mammals in general.

330

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512 FIGURE LEGENDS

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514 Fig. 1. Effects of PCB (118) injection on scale TRAP (a) and ALP (b) activities
515 in goldfish. Each column and the vertical line represent the mean \pm SEM (n = 10
516 samples; one sample from one fish). ** indicates statistically significant
517 difference at $P < 0.01$ from the values in the control.

518

519 Fig. 2. Effects of PCB (118) injection on plasma calcium level (mg/100 ml) in
520 goldfish. Each column and the vertical line represent the mean \pm SEM (n = 10
521 samples; one sample from one fish). ** indicates statistically significant
522 difference at $P < 0.01$ from the values in the control.

523

524 Fig. 3. Effects of PCB (118) administration on TRAP (a) and ALP (b) activities
525 in the scales of goldfish at the 6 h of incubation. Each column and the vertical line
526 represent the mean \pm SEM (n = 10 samples; one sample from one fish).
527 * indicates statistically significant difference at $P < 0.05$ from the values in the
528 control.

529

530 Fig. 4. Effects of PCB (118) administration on TRAP (a) and ALP (b) activities
531 in the scales of goldfish at the 18 h of incubation. Each column and the vertical
532 line represent the mean \pm SEM (n = 10 samples; one sample from one fish). * and
533 *** indicate statistically significant differences at $P < 0.05$ and $P < 0.001$,
534 respectively, from the values in the control.

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538 Fig. 5. Effect of PCB (118) (0.25 ppm) in the expression of osteoclastic markers:
539 cathepsin K (a), TRAP (b), and RANKL (c) mRNAs in the scale. The cathepsin
540 K, TRAP and RANKL mRNA levels were normalized by the β -actin mRNA
541 level. The values of ordinate indicate relative ratio of cathepsin K/ β -actin (a),
542 TRAP/ β -actin (b), and RANKL/ β -actin (c) respectively. Each column and the
543 vertical line represent the mean \pm SEM (n = 10 samples; one sample from one
544 fish). * and ** indicate statistically significant differences at $P < 0.05$ and
545 $P < 0.01$, respectively, from the values in the control.

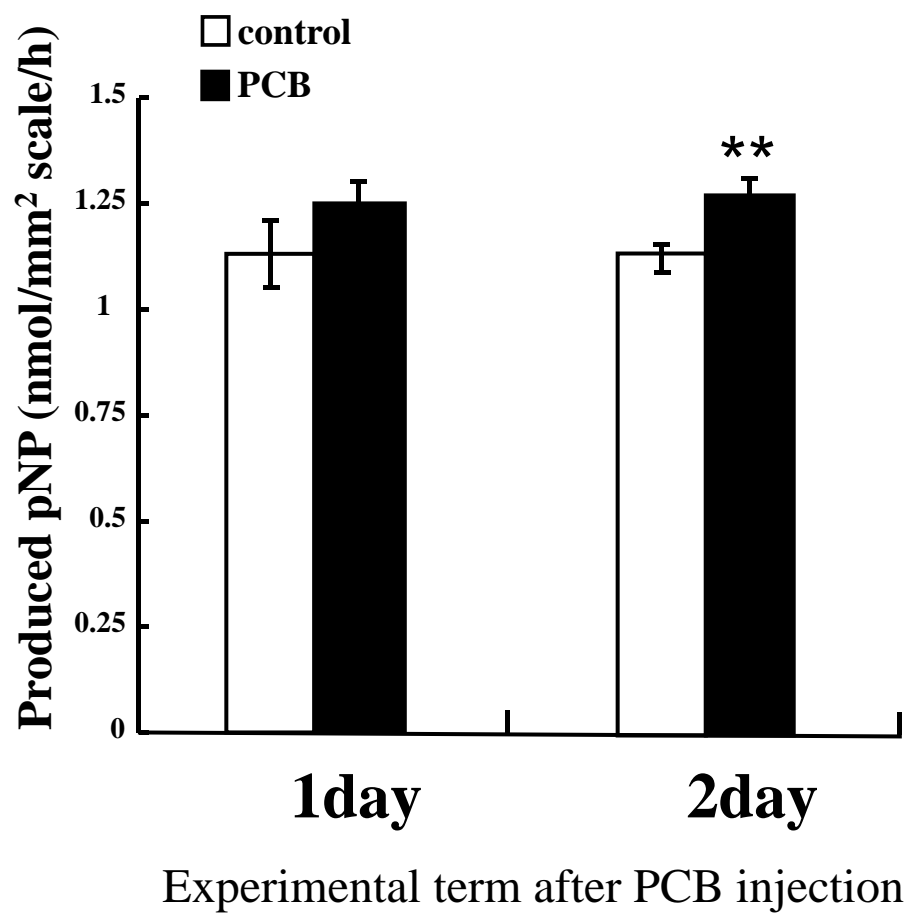
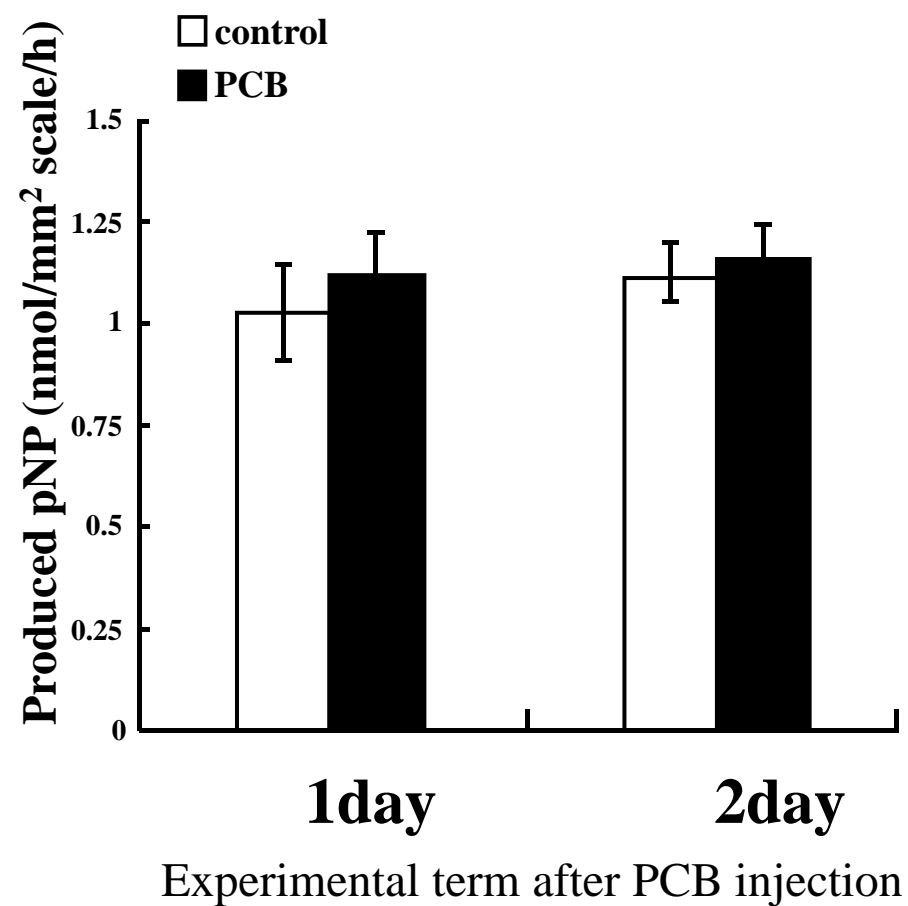
a) TRAP activity**b) ALP activity**

Figure 1 Yachiguchi et al.

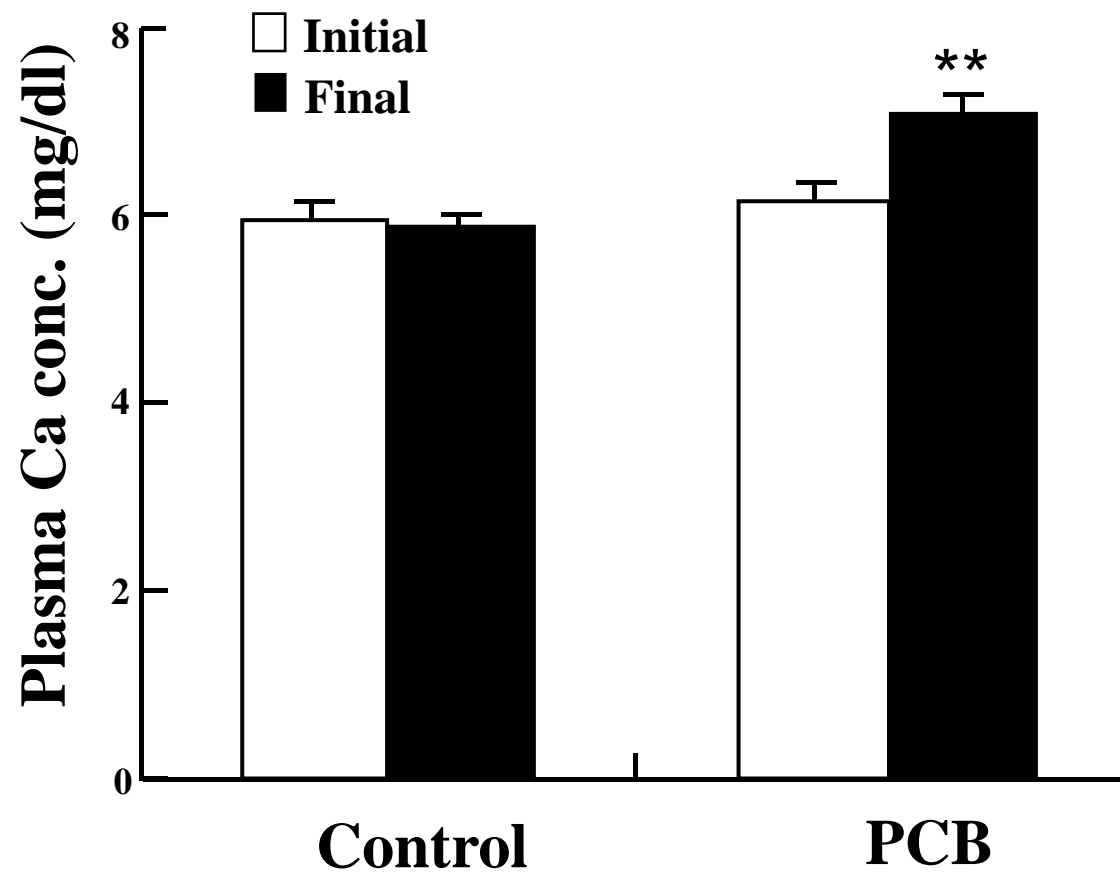
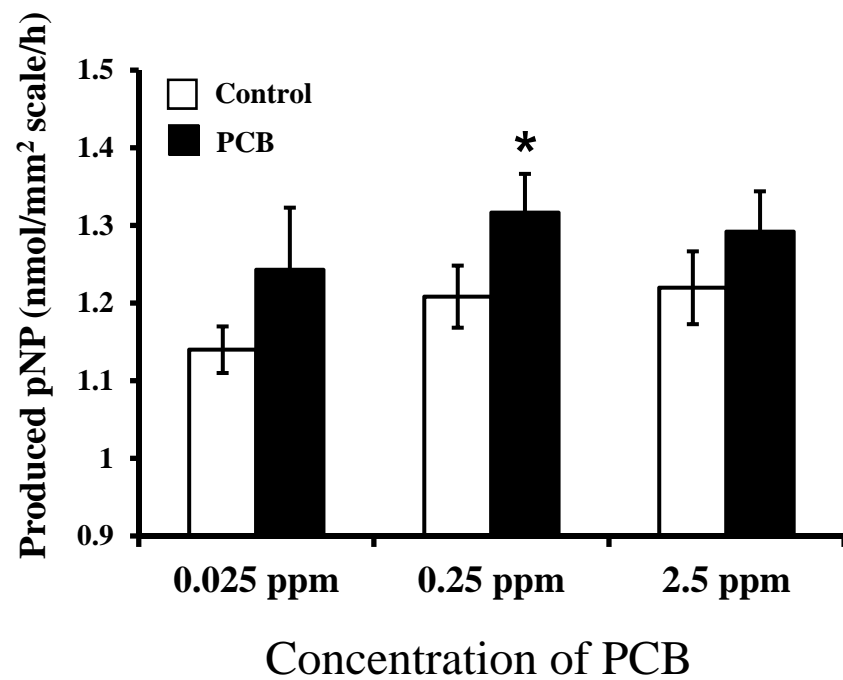


Figure 2 Yachiguchi et al.

a) TRAP activity



b) ALP activity

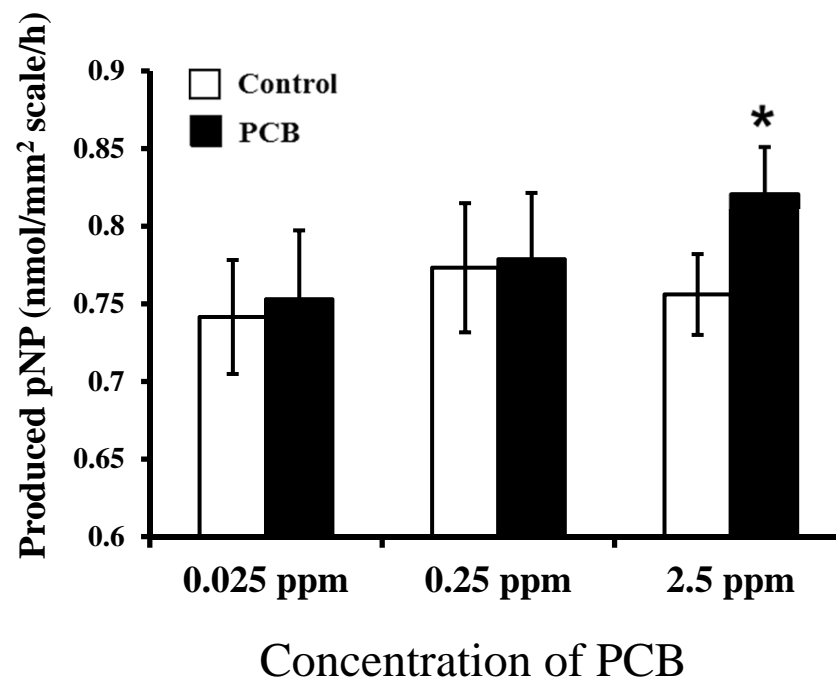
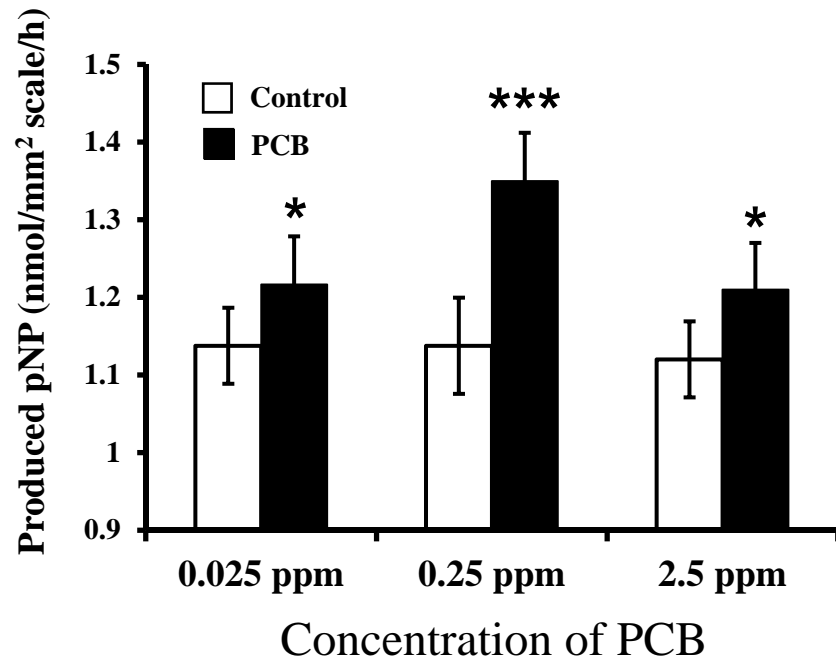


Figure 3 Yachiguchi et al.

a) TRAP activity



b) ALP activity

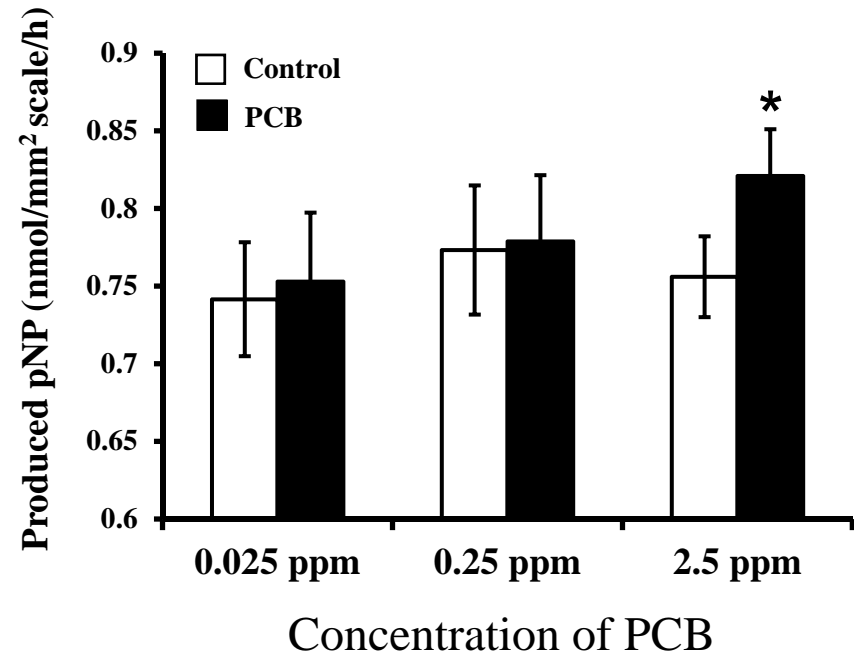


Figure 4 Yachiguchi et al.

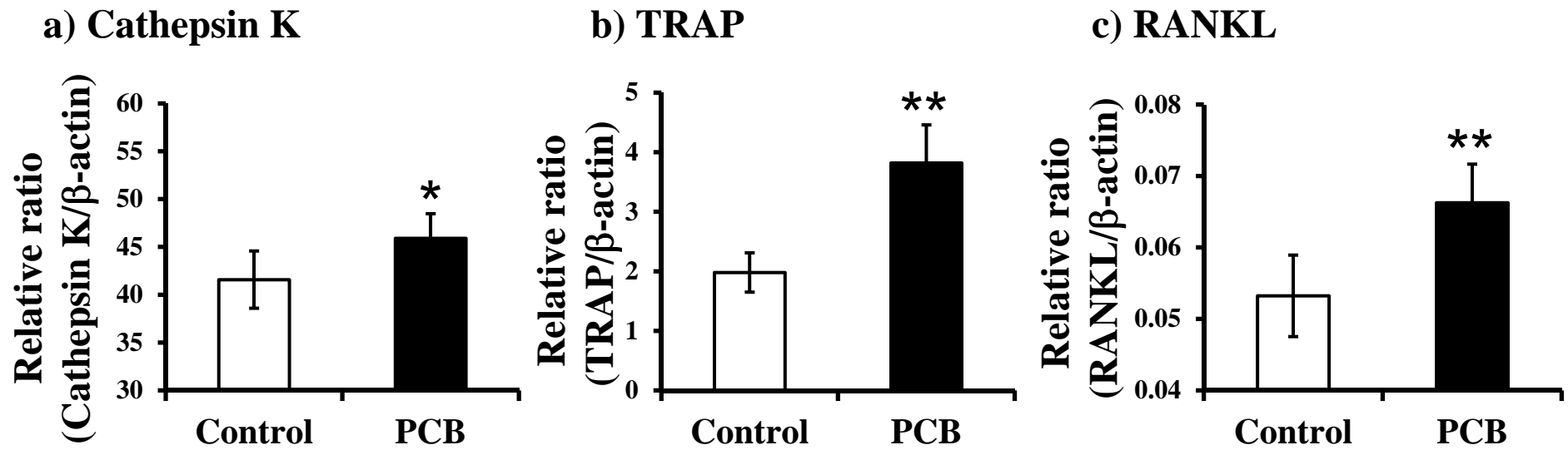


Figure 5 Yachiguchi et al.