Effect of mono-ortho and di-ortho substituted polychlorinated biphenyl (PCB) congeners on leopard frog survival and sexual development

Mariana Beatriz Jofré a,*, William H. Karasov b

a Área de Biología, Departamento de Bioquímica y Ciencias Biológicas, Universidad Nacional de San Luis, Chacabuco y Pedernera, 5700 San Luis, Argentina
b Department of Wildlife Ecology, 221 Russell Labs, 1630 Linden Drive, University of Wisconsin, Madison, WI 53706, USA

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Abstract

We tested the effect of mono-ortho and di-ortho PCB congeners on northern leopard frog (Rana pipiens) hatching success, survival and sexual development. Embryos and tadpoles were exposed to two levels (0.5 and 50 μg/l) of two PCBs. PCBs 101 and 70 were selected because they were present in amphibians collected in the Fox River–Green Bay ecosystem and they have the theoretical structural requirements to be able to bind to the estrogen receptor and mediate estrogenic responses. The exposure of leopard frog embryos and tadpoles to PCB 70 and 101 did not significantly affect hatchability, survival, deformities or growth. There were significant departures from the expected 50:50 sex ratio in tadpoles/froglets exposed to PCB 101 and PCB 70. In all the cases of significant departure, the bias was towards higher number of females. Decrease in the proportion of male gonads and increase in the proportion of intersex gonads were observed with increasing PCB tissue concentrations. The effects of PCB congeners on sexual differentiation occur at concentrations higher than observed in frogs in the Fox River/Green Bay ecosystem.

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Keywords: Leopard frog (Rana pipiens); PCBs; Sexual development; Green Bay–Fox river; Survival

1. Introduction

Polychlorinated biphenyls (PCBs) are a class of synthetic, persistent, lipophilic, halogenated aromatics that are still found in the environment despite their discontinued synthesis and use in many countries (Zhou et al., 2004). In the Fox River/Green Bay ecosystem in Wisconsin, USA, the industrial recycling of carbonless copy paper until 1972 by pulp and paper mills that line the Fox River is linked to the accumulation in sediments of thousands of kilograms of PCBs (Steuer et al., 1995). PCB (mono- and di-ortho PCB congeners, routine PCB congeners and total PCBs) levels in green frog (Rana clamitans) tadpoles and juvenile leopard frogs (R. pipiens) just post-metamorphosis that were raised in enclosures in situ, were correlated with local PCB levels in sediments (Jung, 1996; Karasov et al., 2005) and total PCB levels in adult leopard frogs were positively correlated with concentrations in sediments of wetlands where they were collected (Huang et al., 1999). Low amphibian diversity and hatching success were associated with high contamination in this ecosystem (Jung, 1996; Karasov et al., 2005).

PCBs induce a broad spectrum of toxic and biochemical responses, which are directly related to their molecular structure (McKinney and Waller, 1994; Fielden et al., 1997). The term co-planar is applied to PCB congeners with no substitution at the ortho position (PCBs 77, 81, 138 and 153) and those with one substitution in the meta- or para- position (PCBs 28, 52, 101 and 118). Co-planar congeners, which have high estrogenic activity, are rapidly metabolized to compounds that have low estrogenic activity (Walls et al., 1974). Non-co-planar congeners are rapidly metabolized to non-estrogenic compounds (McKinney and Waller, 1994).
126 and 169). Many of the responses elicited by non-ortho co-planar PCBs correlate with their binding affinity to the Aryl hydrocarbon receptor (AhR) which is believed to mediate several of the effects induced by these compounds (Battershile, 1994; Zhou et al., 2004). Non-co-planar PCBs exhibit low or negligible binding affinity for the Ah receptor due to the presence of ortho chlorine substitution. Some of them are referred to as mono-ortho and di-ortho co-planar (Safe, 1990) because they are direct derivatives of coplanar PCBs and they exhibit some similar responses (Zhou et al., 2004). The majority of ortho substituted PCBs evoke many other responses not related to the Ah receptor mediated mechanism of action, like decrease in dopamine levels, alteration in retinoid and thyroid hormone levels and binding to the estrogen receptor (Safe, 1990; Van den Berg et al., 1998). Many PCBs have shown estrogenic/androgenic activities in vitro (Andersson et al., 1999; Arcaro et al., 1999; Vakharia and Gierthy, 1999; Du et al., 2000). There are some theoretical structural requirements predicted for a PCB in order to be able to bind to the estrogen receptor and hence mediate estrogenic responses. These are restriction about the twist bond due to ortho substitution and also the presence of vacant lateral positions that are amenable to para-hydroxylation. It is postulated that an increase in the ortho chlorine substitution together with the hydroxylation at para positions would lead to estrogenic effects of PCBs (McKinney and Waller, 1994; Fielden et al., 1997).

Sex reversal in response to exogenous estrogens has been described in Xenopus laevis and in several species of ranids (Padoa, 1938; Gallien, 1941; Hsu et al., 1978; Villalpando and Merchant-Larios, 1990; Nishimura et al., 1997), including leopard frogs (Witschi, 1953; Chang and Witschi, 1955; Mackenzie et al., 2003). Although, there are different results about the effects of PCBs on gonadal development, due to different experimental designs, animals and congeners used, in general, it can be concluded that PCBs and/or their metabolites interfere with sexual determination and sex differentiation and induce ovotestes and reversal in animals (Zhou et al., 2004).

In amphibian tissue in the Fox River/Green Bay ecosystem, the proportion of mono- and di-ortho substituted PCBs in tadpoles and frogs was always higher than the proportion of non-ortho congeners (Jung, 1996; Huang et al., 1999; Karasov et al., 2005). Also, the levels of non-ortho PCBs in water and in amphibian tissues are much lower than levels associated with toxic effects in laboratory dose-response studies, such as edema, reduction in growth rate, and mortality (Huang et al., 1998; Rosenshield et al., 1999; Huang and Karasov, 2000; Joferé et al., 2000). The effects of mono- and di-ortho PCBs on amphibians cannot be predicted at present because of a paucity of studies, because relative binding affinity of specific PCB congeners to estrogen receptors may be species specific (Mathews and Zacaharewski, 2000) and because even if binding occurs it can have either an estrogenic or antiestrogenic effect. The objective of this work was therefore to test the effect of ecologically relevant and higher concentrations of mono-ortho and di-ortho PCB congeners on leopard frog hatching success, survival and sexual development. We included sexual development as an endpoint in this study because of the possible estrogenic effect of some of these congeners.

PCB 70 (2,3′,4,5-tetrachlorobiphenyl, a mono-ortho congener) and PCB 101 (2,2′,4,5,5′-pentachlorobiphenyl, a di-ortho congener) were selected because they were present at relatively high concentrations in amphibians collected in the Fox River–Green Bay ecosystem (Karasov et al., 2005) and they also have the theoretical structural requirements for being estrogenic: restriction about the twist bond due to ortho substitution and vacant lateral positions that are amenable to para-hydroxylation (Huang et al., 1999).

We tested three predictions. First, these congeners at levels that occur in the Fox River/Green Bay ecosystem will have no effect on hatchability, survival, or growth, and will not cause edema in frog tadpoles. Second, tadpoles would accumulate PCB congeners 70 and 101, and PCB body burdens after exposure would be similar or lower than after exposure to PCB 126 (Rosenshield et al., 1999). Third, one or more of these congeners will have an effect on sexual development, feminizing in particular, and so we predicted that an increase in PCB concentration would cause a lower proportion of males and either a higher proportion of females or of animals with gonads considered intersex (with both male and female characteristics).

2. Materials and methods

2.1. Study organism

Three leopard frog egg clutches (300 eggs/clutch approximately) were identified and collected by netting in a pond in Sensiba, Brown County, Wisconsin, a site with undetectable sediment levels of PCBs (Karasov et al., 2005). The fourth clutch was laid in the lab from an amplexing pair collected at that pond; eggs from this pair were laid a few hours after collection. These four wild egg clutches were held in pond water in PCB-free plastic containers and transported to the Water Science and Engineering Laboratory at the University of Wisconsin-Madison. Embryos and tadpoles were staged during the experiment following the table proposed by Gosner (1960).

2.2. Exposure of embryos

Each of the four clutches was exposed to the range of PCB concentrations. Eggs were between blastula stage (stage 8) and dorsal lip stage (stage 10) when placed into treatment solutions for the hatchability study. Clutches of eggs (eggs released by one female and fertilized by one male) were kept separate throughout the experiment. Eggs from each of the clutches were exposed to a low (0.5 μg/l) and a high (50 μg/l) dose of two PCBs (congeners 101 and
70, 99.7% and 99% purity, respectively) (Accu Standard, New Haven, CT, USA) and three control treatments. The first control (C+) contained water plus 0.08% acetone (99.9% pure, HPLC grade, Sigma Chemical, St. Louis, MO, USA) as carrier for the PCB, the second control (C−) contained only water. A third treatment of 100 \( \mu g/l \) of 17\( \beta \)-estradiol was added as a positive control for effects on sexual differentiation, because it has been shown to produce sex reversal in leopard frog tadpoles when they are exposed prior to gonadal differentiation (Hsu et al., 1978; Villalpando and Merchant-Larios, 1990; Hayes, 1997; Nishimura et al., 1997), although this concentration is high compared to concentrations that resulted in shifts in the sex ratio of some other anuran species (Pickford et al., 2003; Gallien, 1962). In preliminary studies in our laboratory with \( R. \) pipiens, in an exposure regime similar to the one used in our study, we found that a lower dose (20 \( \mu g/l \) 17\( \beta \)-estradiol) shifted sex ratio towards females in 5 of 7 clutches, whereas the shift occurred in all 7 clutches at 100 \( \mu g/l \) (K. Loeffler, unpublished results). Randomly drawn embryos from each clutch were subdivided into groups of 70 and each of these groups was exposed to 70 ml of one treatment solution in 100 \( \times \) 20 mm glass petri dishes, giving a total of 28 petri dishes (seven treatments, for four egg clutches). Petri dishes were placed into a 23 °C incubator on a 14:10 light:dark cycle. Treatment solutions were changed every 24 h (static renewal system) and were prepared with dechlorinated, charcoal filtered water (pH 8.2, hardness 324 mg/l as CaCO\(_3\), dissolved oxygen 11.5 mg/l).

Embryos were exposed to treatments for four to five days. On the day embryos hatched, hatching success, survival and deformities were recorded.

2.3. Exposure of tadpoles

After all embryos had hatched, 25 surviving tadpoles (Stage 23–24 (Gosner, 1960)) from each petri dish were transferred to glass tanks containing seven liters of the same treatment solutions described in the hatching experiment. Clutches were kept separate throughout the experiment. Tanks (four for each treatment, one for each clutch) were placed in a water bath kept at 23–24 °C and a 14:10 light:dark cycle. Water treatments in tanks were changed and tadpoles were fed every three days. Tadpole food consisted of boiled romaine lettuce blended into a puree and combined with a 3:1 Rabbit Chow:TetraMin mixture (LM Animal Farms, Pleasant Plain, OH, USA; TetraMin Flake Food, TetraSales, Blacksburg, VA, USA). When the front legs of a tadpole emerged, the animal was measured and transferred to a tilted plastic tub containing one liter of treatment solution. Once placed in the tubs, metamorphs were not fed and treatment solutions were changed every three days.

Tadpoles were exposed to treatments for 108 ± 2 days. Tanks were checked every day for mortality and all dead tadpoles were removed and preserved in 10% formalin. Any deformities or abnormal swimming behaviors were recorded daily. From each tank every 10 days we selected eight tadpoles arbitrarily, to minimize systematic error, and measured snout-vent length (SVL), which is the total tadpole length minus tail length, and total length. This continued until the first tadpole with emergent front legs was observed (day 58 after the experiment in tanks started) giving a total of five times during exposure.

At metamorphosis (when tail length < 2 mm), frogs were weighed, measured for snout-vent length, and euthanized by immersion in a MS222 solution (3-aminobenzoic acid ethyl ester: 0.05% solution, Sigma Chemical, St. Louis, MO, USA). Frogs were dissected to determine masses (±0.001 g) of liver, kidneys plus gonads, and fat bodies. Time to metamorphosis for each frog was recorded. Tadpoles that failed to metamorphose by the end of the experiment were weighed, measured for total length, staged, and euthanized. After euthanization, samples of tadpoles from each treatment (clutches pooled; PCB 101: 50 \( \mu g/l \): 17 tadpoles (12.14 g); 0.5 \( \mu g/l \): 16 tadpoles (13.4 g); PCB 70: 50 \( \mu g/l \): 15 tadpoles (12.72 g); 0.5 \( \mu g/l \): 18 tadpoles (13.4 g); controls: 17 tadpoles (12.52 g)) were packed in aluminum foil and frozen for contaminant analysis. Gonads of all metamorphosed frogs and of tadpoles that failed to metamorphose by the end of the experiment were dehydrated in graded ethanol and xylene, paraffinized and embedded in standard embedding cassettes. Serial sections of the tissue blocks were prepared and routine staining (standard hematoxylin and eosin) was performed. Sex was determined by observing the gonad slides in a microscope at 40× magnification. A metamorphosed frog or tadpole was considered: (a) as male, if gonads had seminiferous tubules (Fig. 1a), (b) as female, if both gonads presented only ovocites either with or without nuclei (Fig. 1b) and, (c) intersex, if gonads had ovarian and testicular gonadal tissue in a single gonad, i.e., oocytes present within testicular tissue (testicular ovocites or ovotestis) (Mackenzie et al., 2003; Pickford et al., 2003; Reeder et al., 2005; Coady et al., 2005) (Fig. 1c).

Tadpoles were analyzed for levels of both PCB congeners (#70 and #101) at the Wisconsin State Lab of Hygiene (WSLH), University of Wisconsin-Madison following procedures described in the Laboratory Methods for Organic Analysis (Wisconsin State Laboratory of Wisconsin State Laboratory of Hygiene, 1993).

2.4. Statistical analysis

Proportions of embryos hatching, tadpoles surviving, tadpoles with deformities and tadpoles undergoing metamorphosis from each petri dish/tank, were transformed to arcsin square roots because proportions tend not to be normally distributed. Values of total length of tadpoles were included in the analyses untransformed. Frogs or tadpoles with undetermined sex due to loss or poor preparation of gonads (11.2% of 802 gonads inspected) were
removed from the analysis of sex but were retained in other analyses.

The hatchability study was considered as a separate experiment and analyzed independently of the tadpole exposure study. Analysis of variance (ANOVA) was used to compare features of embryos (hatchability, survival and deformities) among the seven treatments (PCB 101 low and high, PCB 70 low and high, C−, C+, and estradiol).

In the tadpole exposure experiment, where our primary interest was in testing for concentration-dependent effects among the two types of PCBs, we first used analysis of variance (ANOVA) to compare features (survival, deformities, metamorphosis and length) among the three types of control treatments (C−, C+, and estradiol). The statistical model always included a constant and treatment and clutch as factors. Clutch was included in the models as a factor because, in our experience, clutches can be a significant source of variation (i.e., clutches sometimes respond differently to treatments) and tests for treatment effects are more powerful when the clutch effects are accounted for. Second, ANCOVA was used to test for PCB concentration-dependent effects on features (survival, deformities, metamorphosis and length) among the PCB treatments, two PCBs (101 and 70), each at three tissue concentrations. PCB levels were not measured in treatment water, but we confirm exposure levels by measuring tissue PCB concentrations, values that were included as the covariate in the ANCOVA analysis. The statistical model always included a constant, PCB type, and clutch as factors and log[PCB tissue concentration] as the covariate, plus all the possible interactions between factors.

ANCOVA was used to relate log concentration of PCBs in tissues of tadpoles that failed to metamorphose by the end of the experiment to nominal concentrations of treatment water.

Fig. 1. Gonad histology of froglets/tadpoles in the experiment. (a) Normal gonads of males exposed to 0.5 µg/l of PCB 101 (10×). (b) Normal gonad of female exposed to C+ (left: 4×, right: 10×). (c) Gonads (10×) with female and male tissue (intersex) of froglets exposed to 50 µg/l of PCB 70 (left) and 50 µg/l of PCB 101 (right).
Sex ratio data were analysed by replicated goodness of fit test (G-test) (Sokal and Rohlf, 1981), to compare sex ratios with the expected 50:50 male:female ratio, as described by Pickford et al. (2003).

Values for \( p < 0.05 \) for main effects and of \( p < 0.1 \) for interaction terms were considered to be statistically significant. Values for \( p \) for main effects that were \( <0.1 \) and \( >0.05 \) were considered to reflect trends.

3. Results

3.1. Hatching experiment

Treatments (PCB 101 low and high, PCB 70 low and high, C–, C+, and estradiol) did not significantly affect hatchability and survival at hatch (Fig. 2a and b, Table 1). The incidence of deformities was significantly higher for embryos exposed to estradiol (Fig. 2c, Table 1). The deformities observed in newly hatched tadpoles were: body curled up or down, asymmetric body, curled spine, short tail, abnormal tail fins and deformed tail. Edema was not observed in newly hatched tadpoles. Clutch was a significant factor for proportion hatching (Table 1).

3.2. PCB residues in tadpoles

The limit of detection (LOD) was 0.70 \( \mu \)g/kg and the limit of quantification (LOQ) 2.3 \( \mu \)g/kg for both PCBs (101 and 70). The concentration of PCB 101 and PCB 70 in tissues of tadpoles living to the end of the experiment increased in relation to nominal concentration of treatment water (Fig. 3). Low levels of both PCBs were detected in control tadpoles (7.8 ppb of PCB 101 and 15 ppb of PCB 70). The log[PCB concentration in treatment water] was a significant factor in determining log[PCB concentration in tadpole tissues] (\( F_{1,2} = 166.31, p = 0.006 \)). The PCB congener term was not significant (\( F_{1,2} = 0.001, p = 0.975 \)), and neither was the interaction between log[PCB concentration in treatment water] and PCB congener (\( F_{1,2} = 0.779, p = 0.470 \)), indicating that there was no significant difference between the two PCB types in bioconcentration. The bioconcentration factors (BCFs = PCB concentration in wet tadpole tissue/ PCB nominal concentration in treatment water) ranged from 160 to 240 for PCB 101 and from 122 to 194 for PCB 70. The values: 7.8, 120 and 8000 \( \mu \)g/kg for PCB 101 and 15, 97 and 6100 \( \mu \)g/kg for PCB 70, were used as tissue PCB concentrations in ANCOVAs.

3.3. Survival, deformities, growth, and metamorphosis of tadpoles

The three control treatments (C+, C– and 17\( \beta \)-estradiol) did not differ significantly in the proportions of tadpoles that survived or were deformed (Fig. 4a and b), but the proportion of tadpoles that metamorphosed in the

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Proportion hatching</th>
<th>Proportion survival</th>
<th>Proportion deformities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( F )-ratio</td>
<td>( p )</td>
<td>( F )-ratio</td>
</tr>
<tr>
<td>Treatment( a )</td>
<td>0.343</td>
<td>0.905</td>
<td>0.719</td>
</tr>
<tr>
<td>Clutch( b )</td>
<td>5.562</td>
<td>0.007</td>
<td>1.378</td>
</tr>
</tbody>
</table>

\( a \) Treatments (seven) were: PCB congener 101 high (50 \( \mu \)g/l) and low (0.5 \( \mu \)g/l) concentrations; PCB congener 70 high (50 \( \mu \)g/l) and low (0.5 \( \mu \)g/l) concentrations and control treatments: C–, C+ and estradiol (0.1 mg/l).

\( b \) There were four clutches total.
There was no significant effect of PCB type or concentration on survival, or metamorphosis (Fig. 4d and f; Table 2). There was a trend for differences in the incidence of deformities between PCBs (p = 0.092) and for an increase in the incidence of deformities with the increase in tissue PCB concentration (p = 0.070) (Fig. 4c; Table 2). Edema was not observed in tadpoles or metamorphs during the 108 days of exposure to PCB congeners 70 and 101.

Log[PCB tissue concentration] had a significant effect on SVL (Fig. 5) on days 11 (only for PCB 70), 21 and 31 (for both PCBs) after hatch (Fig. 5; p < 0.05 in all cases). On these dates tadpoles from the control treatment were larger than tadpoles exposed to the other two concentrations. These differences in size were not significant later in the study (from day 31).

For some of the parameters (survival, deformities and growth) there were significant differences or trends for differences among the clutches, which underscores the utility of including clutch as a factor (blocking for clutch) to increase the sensitivity of tests for major treatment effects. For example, one clutch (#3) had a significantly lower proportion survival and a significantly higher proportion of deformed tadpoles than the other three clutches (all

![Fig. 3. Relationship between nominal concentration of polychlorinated biphenyls (PCBs) 70 and 101 in treatment water (µg/l) and concentration of PCB 70 and PCB 101 in tadpole tissues (µg/kg wet mass).](image)

17β-estradiol treatment was significantly higher than in the other two treatments (Fig. 4c). The body length and total length of tadpoles did not differ significantly among the three control treatments at any of the times tadpoles were measured.

![Fig. 4. (a) Proportion survival, (b) deformities and (c) metamorphosis of Rana pipiens tadpoles exposed to control treatments and 17β-estradiol. (d) Proportion survival, (e) deformities and (f) metamorphosis of Rana pipiens tadpoles exposed to PCBs.](image)
Also, clutch had a significant effect on lengths on all the dates tadpoles were measured, although there was no apparent pattern of any particular clutch having consistently shorter or longer tadpoles than other clutches.

3.4. Gonadal differentiation of tadpoles

Because gonads from both tadpoles and froglets were checked for determining sex histologically, we first calculate $G$ values for all sex ratio data pooled into pre- and post-metamorph frogs. There was a significant female biased departure from the expected 50:50 sex ratio in both groups (total $G = 58.7$, $p < 0.001$, pooled $G = 58.6$, $p < 0.001$), but this bias was not significantly different between pre- and post-metamorph groups (heterogeneity $G = 0.018$, $p = 0.89$). Therefore, pre- and post-metamorph sex ratio values were pooled to perform $G$ test for individual replicates (clutches) and pooled data (treatments).

When considering pooled sex ratio data (clutches pooled into treatments) there were significant departures from the expected 50:50 sex ratio in all treatments (PCB 101, high and low doses, PCB 70, high and low doses and estradiol; all $p$’s < 0.05), except for the control group ($p = 0.42$). In all the cases of significant departure from the expected sex ratio, the bias was towards higher number of females (Fig. 6). The highest numbers of intersex individuals were observed at the higher doses/body concentrations of both PCBs (16.18% for PCB 101 and 30.36% for PCB 70) (Fig. 6).

When $G$ values were calculated for individual replicates (clutches), significant female bias was detected for clutch 1 at the high and low PCB 101 dose/body concentration and at the low PCB 70 dose/body concentration (Table 3). This significant female biased sex ratio, was also observed for this clutch in the control treatment ($p = 0.046$). Two of the four clutches shown a significant female biased sex ratio, when exposed at the high concentration of PCB 70 (Table 3). Three out of the four clutches had a significant departure from the expected sex ratio after exposure to estradiol, with a higher proportion of females in all clutches (heterogeneity $G = 5.34$, $p = 0.15$) (Table 3).

4. Discussion

Exposure of $R$. pipiens to PCB 101 and PCB 70 during embryo development, did not affect hatchability or survival at hatch. Tadpoles exposed during larval stages accumulated PCB congeners, however tissue levels reached did not affect survival, incidence of deformities and metamorphosis. Significant bias from the expected 50:50 male
A to female sex ratio was observed in tadpoles exposed to PCBs, an effect particularly evident in tadpoles with the higher tissue levels of PCB 70.

PCBs in the laboratory at even higher than environmental concentrations had no significant effect on embryo hatchability (Fig. 2 and Rosenshield et al., 1999). Therefore, although hatchability of leopard and green frog (*Rana clamitans*) embryos in the Fox River/Green Bay ecosystem was negatively correlated with exposure to PCBs (Jung, 1996; Karasov et al., 2005), we doubt that this reflects a direct cause-effect linkage of PCBs per se, although we cannot rule out a direct effect of the sum of PCBs and other contaminants likely correlated with PCBs (such as dioxins and furans). Although, PCBs at environmentally relevant concentrations may negatively affect frog embryo hatchability in combination with high NH₃ (Jofré et al., 2000), leopard frogs in the Fox River/Green Bay ecosystem lay eggs when water temperatures, and hence NH₃ levels, are relatively low (Karasov et al., 2005). Moreover, amphibian eggs have a protective jelly coat that could impede direct contaminant exposure to the developing embryo.

Residues in tadpoles (Fig. 3) confirmed our exposure of developing tadpoles to specific PCB congeners and facilitate the comparison of our results with those from our earlier study on PCB 126 (Rosenshield et al., 1999) and our field studies in the Fox River/Green Bay ecosystem (Jung, 1996; Karasov et al., 2005). As regards the first comparison, we dosed leopard frog embryos and tadpoles in a similar range of concentrations of PCBs as the earlier study (0.05–50 µg/l), and the residue levels that we measured

### Table 3
G-test statistics for comparison of the expected 50:50 sex ratio of tadpoles/froglets exposed to PCBs during 108 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Replicate (clutch)</th>
<th>Intersex</th>
<th>Male</th>
<th>Female</th>
<th>G value</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB 101 High dose/body concentration</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>17</td>
<td>13.553</td>
<td>1</td>
<td>0.00023</td>
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<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>8</td>
<td>0.6986</td>
<td>1</td>
<td>0.40326</td>
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<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>0.1113</td>
<td>1</td>
<td>0.73862</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>9</td>
<td>7</td>
<td>0.2507</td>
<td>1</td>
<td>0.61661</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
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<td>14.613</td>
<td>4</td>
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<td></td>
<td><strong>Pooled</strong></td>
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<td>0.02327</td>
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<td></td>
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<td></td>
<td>9.4652</td>
<td>3</td>
<td>0.0237</td>
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<tr>
<td>PCB 101 Low dose/body concentration</td>
<td>1</td>
<td>0</td>
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<td>15</td>
<td>11.252</td>
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<tr>
<td></td>
<td><strong>Total</strong></td>
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<td>PCB 70 High dose/body concentration</td>
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<td>19</td>
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take longer to depurate than measured for PCB 126 in leopard and green frogs (range 0.0078–8 µg/g wet mass) (et al., 1995; Niimi, 1996). The highest tissue concentrations in these laboratory experiments are well above the highest total PCB concentration measured in either adults collected at polluted sites in the Fox River/Green Bay ecosystem (0.154 µg/g wet mass, (Huang et al., 1999)) or in tadpoles raised in enclosures in situ at polluted sites in that ecosystem (0.57 µg/g wet mass; (Karasov et al., 2005)). Total PCB levels in the most contaminated frogs collected in the Fox River/Green Bay ecosystem correspond to tissue concentrations in the laboratory experimental frogs exposed to 0.5 µg/l (Fig. 3). Thus, the range of exposures to specific PCB congeners in the laboratory experiments brackets the range of exposures in total PCBs in frogs in the Fox River/Green Bay ecosystem.

As we predicted, exposure of leopard frog embryos and tadpoles to PCB 70 and 101 did not significantly affect survival or growth, although there was a trend for a negative effect of log[PCB tissue concentration] on deformities (Fig. 4e). PCB 126 is relatively more toxic to leopard frogs, as exposure to 50 µg/l caused 100% mortality and significantly increased the incidence of edema (Rosenshields et al., 1999). The congeners tested in this experiment are not coplanar and are not mono-ortho or di-ortho derivatives of coplanar PCBs, which can be considered as the most potent in eliciting adverse responses in body weight, lymphoid system function and development, immunotoxicity, and teratogenicity (Safe, 1990). So, it is not surprising to observe an absence of adverse effects, at the same concentrations that PCB 126, a coplanar PCB, has been shown to elicit toxic responses (mortality, and edema). Non-coplanar PCBs, such as the congeners used in the present study, do not exhibit binding affinity for the Ah receptor (AhR), which is believed to mediate several of the effects induced by coplanar PCBs (Battershill, 1994).

PCB 70 and PCB 101 are non coplanar ortho substituted PCB congeners with vacant para positions with unsubstituted carbon atoms susceptible to para hydroxylation, so they conform to the structural requirements necessary for binding to the estrogen receptor (Huang et al., 1999). We predicted that in our test, PCBs would decrease the fraction of males in the study population and results were consistent with this prediction. When the number of intersex individuals were included in the total number of tadpoles, the proportion of females was similar in all treatments (between 54% in tadpoles exposed to the high dose of PCB 101 and 61% at the low dose of PCB 101), except for estradiol; therefore, the departure from the 50:50 expected sex ratio was due to a decrease in the proportion of males and it seemed that most instances of sex reversal involved conversion of the male gonad to one with presence of both seminiferous tubules and oocytes in the same gonad, i.e., intersex. We did not observe any individuals with one male gonad and one female gonad. In other analogous studies of frog PCB exposure, Xenopus laevis exposed to 10 µg/l of PCB3 and PCB2 (mixtures similar to Aroclor 1242 and 1245, respectively) up to the completion of metamorphosis, exhibited a non-significant decline in the incidence of males, but testes from more than a third of male frogs exhibited feminization to different degrees at gross morphology and histology, with fewer or abnormal spermatagonia and oocytes, besides more than 70% of forelimb malformations (Qin et al., 2003, 2005).

Other studies have related environmental PCB levels to sex reversal in frogs. Reeder et al. (1998) found a striking sex ratio reversal in juvenile cricket frogs at sites contaminated with PCBs and PCDFs in Illinois. In a more recent study, historical and geographical trends of intersexuality of cricket frogs in Illinois were also correlated with environmental contamination (Reeder et al., 2005). In laboratory studies abnormal gonads in females and alteration of sex ratios was observed in rainbow trout exposed to Aroclor 1260 (Matta et al., 1998) and sex reversal was reported in turtles exposed to hydroxylated PCBs (Bergeron et al., 1994).

Although congeners tested in this study conform to the structural requirements necessary for binding to the estrogen receptor and tadpoles with the higher tissue PCB levels, particularly of PCB 70, shown a significant female biased sex ratio and the highest number of intersex individuals, we are not able to identify the mechanism of action of induction of intersex by the PCBs in this study. The mechanisms of steroid action on amphibian gonads are not clearly understood (Pickford et al., 2003). Amphibian gonads are able to respond to steroids at times when other tissues lack sensitivity to them but, no estrogen receptors have been identified in amphibian gonads (Hayes, 1998). Exogenous estradiol can also increase the incidence of intersex depending on the window of development during which tadpoles are exposed to it (Villalpando and Merchant-Larios, 1990); although our study exposure was constant throughout development. Furthermore, gonadal differentiation in amphibians may be also regulated by thyroid hormones (Hayes, 1998). Hydroxylation is important for PCBs’ interaction with ER and there is also evidence for estrogene receptor-independent effects of PCBs (Kestner, 2000; Zhou et al., 2004).

Some species/populations of Rana display a stage of natural juvenile hermaphroditism during larval development that may resolve to definitive testes and ovaries by the end of metamorphosis (differentiated breeds) or after metamorphosis (semi-differentiated breeds) (Witschi, 1929, 1930; Eggert, 2004). Although some studies have detected intersex gonad in control groups of R. pipiens, other surveys failed to detect testicular oogenesis or hermaphroditism in metamorphs of this species. Moreover, variation within species both at the population and
subpopulation level has been suggested (Hayes et al., 2003; Mackenzie et al., 2003). If tadpoles exposed to PCBs in this study belong to a differentiated group, the observed effect would be a feminization of males. The occurrence of a few intersex individuals in control treatments, even in post-metamorphs, may indicate that tadpoles in the study belong to a semi-differentiated group (Mackenzie et al., 2003), and the effect of PCB exposure would be a delay of normal intersex individuals in turning into males.

Some other environmental contaminants have been reported to affect gonadal differentiation in amphibians, although data are not always consistent. Bisphenol A significantly altered sex ratio in Xenopus laevis (Kloas et al., 1999), but, at similar concentrations in a different experiment, had no observable effect on sexual differentiation (Pickford et al., 2003). Dibutyl phthalate and styrene induced gonadal feminization in Rana rugosa (Ohtani et al., 2000, 2001). Results regarding the effect of atrazine on gonadal development are also not definitive (cf. Hayes et al., 2002; Coady et al., 2005). Although, our results with PCBs suggest that their effects on sexual differentiation occur at concentrations higher than observed in frogs in the Fox River/Green Bay ecosystem, effects could be possible at environmentally relevant levels of atrazine (Hayes et al., 2002).

5. Conclusion

Hatchability of frog embryos in the Fox River/Green Bay ecosystem has been negatively correlated with natural exposure to PCBs, but PCB congeners 70 and 101, present in amphibians collected in the ecosystem, had no significant effect on Rana pipiens embryo hatchability, at even higher than environmental concentrations.

Tadpoles exposed through embryo and larval development accumulated PCBs, reaching tissue concentrations well above the highest total PCB concentration measured in either adults collected at polluted sites in the Fox River/Green Bay ecosystem or in tadpoles raised in enclosures at polluted sites in that ecosystem. However, those tissue levels did not significantly affect survival or growth, although there was a trend for a negative effect on deformities.

PCB 70 and PCB 101 conform to the structural requirements necessary for binding to the estrogen receptor and, as predicted, affected sex proportions of leopard frogs exposed through larval development. Decrease in the proportion of male gonads and increase in the proportion of intersex gonads were observed with increasing PCB tissue concentrations although the effects of PCB congeners observed on sexual differentiation occurred at concentrations higher than observed in frogs in the Fox River/Green Bay ecosystem.

Acknowledgments

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