Differential effects of testosterone and 17β-estradiol on gonadal development in five anuran species

Rafał P Piprek, Anna Pecio, Jacek Z Kubiak1,2 and Jacek M Szymura

Department of Comparative Anatomy, Institute of Zoology, Jagiellonian University, Gronostajowa 9, 30-387 Krakow, Poland, 1CNRS, UMR 6290, Cell Cycle Group, Institute of Genetics and Development of Rennes, F-35043 Rennes, France and 2UEB, IFR 140, Faculty of Medicine, Université Rennes 1, F-35043 Rennes, France

Correspondence should be addressed to R P Piprek; Email: rafal.piprek@uj.edu.pl

Abstract

Sex hormones are essential for sexual differentiation and play a key role in the development of gonads in amphibians. The goal of this study was to evaluate the influence of exogenous sex steroids, testosterone, and 17β-estradiol (E2) on development of gonads in five anuran species differing in their evolutionary positions, sex determination, and mode of gonadogenesis. We found that in two closely related species of fire-bellied toad, Bombina bombina and Bombina variegata, testosterone and E2 exposure results in sex reversal as well as intersex and undifferentiated gonads. Similarly, sex reversal was observed in Hyla arborea after exposure to male or female sex steroids. Xenopus laevis was sensitive to E2 but only moderately to testosterone. In Bufo viridis, treatment with either sex hormone provoked a developmental delay in gonads and Bidder’s organs. Therefore, susceptibility to hormonal sex reversal appeared species dependent but unrelated to genetic sex determination and the type of gonadogenesis. We also found that the onset of sex steroid exposure influences gonad differentiation and the meiotic status of the germ cells depends on their location within the gonad. Our findings reveal differential sensitivity of amphibians to testosterone and E2, establishing a hierarchy of sensitivity to these hormones among different anuran species.

Introduction

An individual’s sex is defined by a set of specific features that characterize male and female individuals. In some animals such as mammals, birds, amphibians, many reptiles, some fishes, and insects, the development of primary sex features (testes or ovaries) is established genetically (Morrish & Sinclair 2002, Piprek 2009a). In other animals, sex can be determined by temperature or various external factors (Coe 1936, Bull 1980, Dournon et al. 1990, Warner & Shine 2008). In vertebrates, the differentiated gonads produce steroid hormones that orchestrate the development of secondary (genitals) and tertiary (body shape and larynx) sexual features. Further, exogenous sex steroids may exert an impact on gonad development in some animals. This effect was first described by Lillie (1917) in freemartin cattle, where female infertility, due to partial sex reversal, was caused by steroid hormones produced by the opposite-sex twin in the womb. Because abnormalities in hormone levels can have broad effects on sexual development including sex reversal usually accompanied by infertility in mice and humans (Piprek 2009b), sex steroid synthesis, action, and metabolism must be strictly controlled during development.

In amphibians, sex is determined genetically, although it is known that external factors also have an important impact (Hayes 1998). Thus, the sex determination of amphibians is not tightly regulated and is evolutionally malleable. In addition, there are species-dependent differences in the reaction to external stimuli upon sex differentiation, which may be responsible for different sensitivity of various amphibian species to environmental cues (Withgott 2002). Aquatic contaminants such as herbicides act as hormone disruptors and may be involved in global decline of amphibian population in recent years (Collins & Storfer 2003, Murphy et al. 2006). In amphibians, steroid hormones (testosterone and 17β-estradiol (E2)) added to water during tadpole development can, depending on the dose and species, alter gonadal sex (Wallace et al. 1999). Complete sex reversal observable as a one-sex progeny can be interpreted as a major effect caused by high dose of sex steroids; however, weak hormonal effects include partial sex reversal manifested by hermaphroditic (intersex) gonads termed ovotestes (Wallace et al. 1999). Impairment of sexual differentiation was also observed in gonadal culture in vitro (Miyata & Kubo 2000). Early studies suggested that the heterogametic sex is impervious to steroid hormones (Witschi et al. 1985a,
1958b, Gallien 1974). Thus, amphibians with heterogametic males (XY), such as ranids and hylids, should be masculinized by testosterone and insensitive to E2 (Gallien 1950, Takahashi 1958, 1959). On the other hand, some anurans and urodèles with heterogametic females (ZW) should be feminized by E2 and refractory to testosterone (Chang 1955, Chang & Witschi 1955). Later studies have shown that this claim was based in a few species and appeared oversimplified (Kawamura & Nishioka 1977).

In early experiments by Gallien (1954), the administration of steroid hormones to the fire-bellied toads Bombina variegata and Bombina orientalis (Bombinatoridae) did not alter their sex. The author concluded that these species are refractory to sex steroids. However, our recent studies (Piprek et al. 2010) showed that sexual differentiation of B. variegata gonads occurs much earlier in development than in the majority of studied anurans, i.e. at 33 Gosner stage, suggesting that the sex reversal in Bombinatoridae can be induced, provided the hormones are applied sufficiently early in development. Such a hypothesis was in agreement with the data showing that exogenous steroid hormones are able to alter the sex fate of the gonad only if given at a specific time during gonadal development (Villalpando & Merchant-Larios 1990). For example, in Xenopus laevis, sex hormones cause complete sex reversal when administered before sexual differentiation of undifferentiated gonads into ovaries or testes (Villalpando & Merchant-Larios 1990). However, when the hormones are applied during sexual differentiation, they cause partial sex reversal and hermaphroditism, but they have no effect when administered later. Similar phenomena were also described for Rana curtipes and Rana pипiens (Saidapur et al. 2001, Hogan et al. 2008).

Anurans have three different types of gonadogenesis (Ogielska & Kotusz 2004): basic (most species, Xenopus, and Hyla), retarded (genus Bufo – sexual differentiation of gonads around metamorphosis), and accelerated (genus Bombina – sexual differentiation of gonads early about Gosner stage 30). It is possible that a high variation of effects of exogenous factors on the gonads might depend on these types of gonad development.

The aim of this study was to reevaluate the previous findings on the influence of sex steroids (testosterone and E2) on gonadal development in five selected species of anurans. We chose species from separate branches of anuran evolution that differ in their heterogametic status and developmental timing of gonad differentiation. The fire-bellied toads Bombina bombina and B. variegata (Bombinatoridae) were chosen because of the contradictions described earlier (Gallien 1974, Piprek et al. 2010). Hyla arborea (Hylidae), Bufo viridis (Bufonidae), and X. laevis (Pipidae) gonads were studied in parallel because of the high degree of divergence of these amphibian clades (Roelants et al. 2007). In addition, because B. viridis (Bufonidae) also has a specific additional ovary-like structure, the Bidder’s organ, we compared the effect of the sex hormones between this organ and the gonads.

## Results

### B. bombina and B. variegata

Sex steroids had similar effects on the fire-bellied toads B. bombina and B. variegata when exposure was initiated at the same developmental time points in each species (Table 1). The gonadal structure in all individuals exposed to sex steroids deviated from the control (Fig. 1A and D). We observed a wide range of E2 effects, from underdeveloped testes and ovaries to undifferentiated gonads and intersex gonads (Table 2). The intersex gonads (ovotestes) and undifferentiated gonads appeared only when E2 was administered just after hatching and at the beginning of the gonadal differentiation (groups I_E and II_E). The number of individuals with ovaries predominated over the rest in group I_E (up to 57.9% of individuals; Table 2). Thus, the sex ratio was distorted indicating the sex reversal (M:F 0.7 in B. bombina and 0.18 in B. variegata in group I_E; P<0.05; Table 2). The signs of developmental inhibition were visible in all gonads from groups I_E; and II_E.

A retardation of ovarian development was manifested by relatively thin cortex and the medulla without the secondary cavity at the stage of metamorphosis (i.e. Gosner stage 47; Fig. 1B). The testes were present in some E2-treated tadpoles, and the total number of males (up to 31.8%) in this group was reduced in comparison with control (P<0.05; Table 2). Such testes were always underdeveloped, and during metamorphosis, they

### Table 1

<table>
<thead>
<tr>
<th>Sampling points</th>
<th>Bombina bombina</th>
<th>Bombina variegata</th>
<th>Hyla arborea</th>
<th>Bufo viridis</th>
<th>Sampling points</th>
<th>Xenopus laevis</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>6</td>
<td>4</td>
<td>14</td>
<td>20</td>
<td>49</td>
<td>8</td>
</tr>
<tr>
<td>31</td>
<td>4</td>
<td>7</td>
<td>26</td>
<td>26</td>
<td>52</td>
<td>8</td>
</tr>
<tr>
<td>34</td>
<td>6^a</td>
<td>8^a</td>
<td>34^a</td>
<td>19</td>
<td>54</td>
<td>7^a</td>
</tr>
<tr>
<td>38</td>
<td>9</td>
<td>13</td>
<td>18</td>
<td>73</td>
<td>58</td>
<td>15</td>
</tr>
<tr>
<td>44</td>
<td>18</td>
<td>26</td>
<td>72</td>
<td>53^a</td>
<td>61</td>
<td>30</td>
</tr>
<tr>
<td>47</td>
<td>81</td>
<td>45</td>
<td>159</td>
<td>109</td>
<td>66</td>
<td>113</td>
</tr>
<tr>
<td>Sum</td>
<td>124</td>
<td>103</td>
<td>323</td>
<td>300</td>
<td>Sum</td>
<td>181</td>
</tr>
</tbody>
</table>

^aSampling points at which first signs of sexual differentiation of gonads were visible.
resembled testes of individuals at Gosner stage 38 of the control group in which small testicular cords were filled with primary and secondary spermatogonia (Fig. 1 E). Importantly, in group II E, intersex gonads visibly predominated (i.e. 40% in *B. bombina* and 66.67% in *B. variegata*; Table 2). Such ovotestes were characterized by the presence of germ cells in both medulla and cortex (Fig. 1 C). The meiotic germ cells in ovotestes were present exclusively in the cortex. In some individuals from group IE (13.64% in *B. bombina* and 21.05% in *B. variegata*), gonads remained undifferentiated at the completion of metamorphosis. Such gonads contained germ cells only at the gonial stage. When tadpoles were exposed to E2 at a later time (starting from Gosner stage 40, group III E), no hermaphroditic or underdeveloped gonads were found and the sex ratio indicated a lack of sex reversal (Table 2).

In testosterone-treated tadpoles from groups I T and II T, gonads were reduced to small remnants either totally lacking germ cells or containing a few germ cells surrounded by a few somatic cells (Fig. 1 F). Such reduced gonads predominated in group I T (94.11% in *B. bombina* and 100% in *B. variegata*; Table 3). In group II T, the testes and intersex gonads predominated in *B. variegata* (Table 3), and these gonads did not deviate from the testes and ovotestes of tadpoles exposed to E2. The higher rate of females in group III T may result from a relatively low number in experimental test.

**X. laevis**

The exposure of *X. laevis* tadpoles to E2 at the beginning of hatching (group I E; Fig. 2B) resulted in a significant degree of feminization manifested by high predominance of ovaries (in 94.29% animals). At Nieuwkoop stage 54, the germ cells in the ovaries were secondary oogonia and diplotene oocytes (Fig. 2B) whereas only primary oogonia were observed at the same stage in the

---

**Figure 1** Effects of sex steroids on gonadal development in *Bombina bombina*. (A) At Gosner stage 38, structure of the control ovary revealed germ cells (arrows) located within the cortex (C). The sterile medulla (M) filled the central part of the gonad. (B) During the metamorphosis, the ovaries of estradiol-exposed animals from group I E seemed retarded due to the lack of the secondary cavity in the medulla (M) and the relatively thin cortex (C). Nevertheless, both oogonia (Og) and oocytes (Oc) were visible in the cortex. (C) During metamorphosis, in some individuals, the estradiol treatment resulted in the formation of intersex gonad. In such a gonad, germ cells were located in both the cortex (C) and the medulla (M). Germ cell in meiosis (asterisk) within the cortex. (D) In the control male gonad, germ cells (mainly secondary spermatogonia (Sg)) were located only within the medulla, while the cortex (arrow) was thin and usually indiscernible at Gosner stage 38. (E) After estradiol treatment, some testes were slightly altered during the metamorphosis. Signs of underdevelopment were discernible. Spermatogonia (Sg) were scattered among somatic cells (arrow). (F) Testosterone caused the retardation of gonadal development and such gonads consisted only a few germ and somatic cells clusters (arrowhead) attached to the wall of the vena cava during the metamorphosis. Scale bar 30 μm. *Very similar results were obtained in *B. variegata*. 

---

*www.reproduction-online.org*
control (Fig. 2A). In the control, diplotene oocytes appeared at Nieuwkoop stage 58, which indicated an acceleration of meiosis as a result of E2 exposure. The rest of the gonads were classified as undifferentiated (5.71%; Table 2). Such gonads were underdeveloped and contained a thin cortex and a small medulla without a cavity. Among the tadpoles exposed to E2 starting from the period of gonadal sexual differentiation, i.e., Nieuwkoop stage 51 (group IIIE), ovaries predominated (85.71%), but occasional testes were also observed (9.52%). Such testes were visibly reduced in size and contained only a few testicular cords (Fig. 2E); the development of testes was impaired, manifested by a decreased number of testicular cords, and the presence of underdeveloped rete testis at metamorphosis (Fig. 3E). The rest of the gonads were classified as undifferentiated (9.30% in group IE and 8.51% in group II E; Table 2). A few individuals had undifferentiated gonads (3.77% in group IE and 2.13% in group II E; Table 2). The ovaries in tadpoles exposed to E2 from hatching (group IIE) contained primary and secondary oogonia as well as leptotene and diplotene oocytes, as in the control (Fig. 3A and B). However, their medulla was underdeveloped and the cavity was absent in the whole gonad. The development of testes was impaired, manifested by a decreased number of testicular cords, and the presence of underdeveloped rete testis at metamorphosis (Fig. 3E). Only primary spermatogonia were visible within the testicular cords at this period, as in the control (Fig. 3D).

**Table 2** Effect of estradiol on sex ratio among five anuran species.

<table>
<thead>
<tr>
<th>Sp.</th>
<th>O</th>
<th>Survived</th>
<th>Testes</th>
<th>Intersexual</th>
<th>Ovaries</th>
<th>Undifferentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td><em>Bombina</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bombina</em></td>
<td>IIIE 44</td>
<td>40</td>
<td>37</td>
<td>47.17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bombina</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>variegata</em></td>
<td>IIIE 44</td>
<td>40</td>
<td>37</td>
<td>47.17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Xenopus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>laevis</em></td>
<td>IIIE 44</td>
<td>40</td>
<td>37</td>
<td>47.17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hyla</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>arborea</em></td>
<td>IIIE 44</td>
<td>40</td>
<td>37</td>
<td>47.17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bufo</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>viridis</em></td>
<td>IIIE 44</td>
<td>40</td>
<td>37</td>
<td>47.17</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

n. Number; O. onset of experiment; Sp., species.

Significant difference from the control in the number of males and females (χ² test, P<0.05).

In *H. arborea*, tadpoles exposed to E2 starting from hatching and Gosner stage 34 (groups IIE and IIIE) did not exhibit changes in sex ratio (e.g., 47.17% of females and 43.4% of males in group IIE, P<0.05; Table 2), but some individuals with intersex gonads were found (5.66% in group IIE and 8.51% in group IIIE; Table 2). A few individuals had undifferentiated gonads (3.77% in group IE and 2.13% in group IIIE; Table 2). The ovaries in tadpoles exposed to E2 from hatching (group IIE) contained primary and secondary oogonia as well as leptotene and diplotene oocytes, as in the control (Fig. 3A and B). However, their medulla was underdeveloped and the cavity was absent in the whole gonad. The development of testes was impaired, manifested by a decreased number of testicular cords, and the presence of underdeveloped rete testis at metamorphosis (Fig. 3E). Only primary spermatogonia were visible within the testicular cords at this period, as in the control (Fig. 3D).

Testosterone exposure beginning at hatching (group IIE) or Gosner stage 34 (group IIIE) caused a reduction in the number of individuals with ovaries together with an increase in the percentage of intersex gonads (9.30% of intersex in group IIE and 18.18% in group IIIE; Table 3). Intersex gonads were also observed when testosterone was administered at Gosner stage 34 (18.18% in group IIIE; Table 3). In such gonads, some germ cells entered meiosis and thus preleptotene and leptotene...
oocytes were present during metamorphosis. Diplotene oocytes were never observed in intersex gonads. Importantly, the meiotic cells were located only in the cortex. In the ovaries, diplotene oocytes were visible in the cortex and the medulla was well separated; however, the secondary cavity was absent. The testes of tadpoles treated with testosterone showed a delay of development in comparison with control testes (Fig. 3D and F). Such male gonads were smaller than those in control tadpoles and contained visibly fewer testicular cords. The rete testis appeared normal in comparison with E2-treated tadpoles (Fig. 3E and F).

Sex hormone treatment beginning from Gosner stage 34 (groups II) gave similar results to the exposure described for groups I (Tables 2 and 3; $P < 0.05$). In contrast, the gonads in tadpoles treated with sex steroids starting from stage 40 (groups III) resembled the controls (Fig. 3A and D; $P < 0.05$). There was no detectable impairment of gonad development in this group, and the male to female ratio was unaffected (M:F 0.95 in groups III and IV; $P < 0.05$).

**B. viridis**

In the E2-treated tadpoles, the development of gonads was highly altered in groups I and II. In group I, the majority of individuals (59.26%) had undifferentiated gonads (Fig. 4B), which resembled undifferentiated control gonads at Gosner stage 34 with small medulla and germ cells located in the cortex. The ovaries were found in 18.52% of individuals from group I and the testes were present in 22.22% of individuals. Their testicular cords were visibly smaller at the period of metamorphosis. Intersex gonads were found only when tadpoles were exposed from Gosner stage 42 (48.15% in group II but were also observed in a single control individual. Such ovotestes after E2 treatment were composed of well-differentiated cortex and medulla and germ cells located in both of these layers (Fig. 4E). In the cortex, gonial cells and meiotic cells were discernible whereas in the medulla only gonial cells were seen. The meiotic cells in the cortex resembled preleptotene oocytes. In the Bidder's organ, oogenesis was inhibited only in group I (Fig. 4H) but was not altered in other groups, perhaps because the structure of this organ was established at Gosner stage 37. During metamorphosis, meiocytes in preleptotene phase were visible in this anterior part of the gonad but no diplotene oocytes were present, whereas in the control, the Bidder's organ was filled exclusively with vitellogenic oocytes (Fig. 4G).

Testosterone treatment resulted in drastic inhibition of gonadal development. The majority of gonads in testosterone-treated tadpoles (89.74% in group I) was undifferentiated during metamorphosis and resembled control genital ridges at the earliest stage (Gosner stage 29; Fig. 4C). In some individuals (2.56% in group

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Stage</th>
<th>Number</th>
<th>Testes</th>
<th>Ovaries</th>
<th>Intersex</th>
<th>Undifferentiated</th>
<th>Controls</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bombina bombina</strong></td>
<td>IT hatching</td>
<td>20</td>
<td>17</td>
<td>5.86</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>IIT</td>
<td>34</td>
<td>18</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>IIIT</td>
<td>40</td>
<td>7</td>
<td>100</td>
<td>3</td>
<td>42.86</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>IV control</td>
<td>20</td>
<td>20</td>
<td>100</td>
<td>11</td>
<td>55</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td><strong>Bombina variegata</strong></td>
<td>IT hatching</td>
<td>20</td>
<td>14</td>
<td>70</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>IIT</td>
<td>34</td>
<td>18</td>
<td>83</td>
<td>2</td>
<td>66.67</td>
<td>10</td>
<td>66.67</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>IIIT</td>
<td>40</td>
<td>6</td>
<td>100</td>
<td>2</td>
<td>33.33</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>IV control</td>
<td>18</td>
<td>17</td>
<td>94.44</td>
<td>8</td>
<td>47.06</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td><strong>Xenopus laevis</strong></td>
<td>IT hatching</td>
<td>40</td>
<td>37</td>
<td>92.50</td>
<td>16</td>
<td>43.24</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>IIT</td>
<td>51</td>
<td>20</td>
<td>100</td>
<td>7</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>IIIT</td>
<td>56</td>
<td>20</td>
<td>100</td>
<td>10</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>IV control</td>
<td>20</td>
<td>18</td>
<td>90</td>
<td>9</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td><strong>Hyla arborea</strong></td>
<td>IT hatching</td>
<td>50</td>
<td>43</td>
<td>86</td>
<td>22</td>
<td>51.16</td>
<td>4</td>
<td>9.30</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>IIT</td>
<td>34</td>
<td>45</td>
<td>97.50</td>
<td>20</td>
<td>45.45</td>
<td>19</td>
<td>48.72</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>IIIT</td>
<td>40</td>
<td>40</td>
<td>100</td>
<td>19</td>
<td>51.28</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>IV control</td>
<td>40</td>
<td>37</td>
<td>92.50</td>
<td>8</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td><strong>Bufo viridis</strong></td>
<td>IT hatching</td>
<td>45</td>
<td>39</td>
<td>86</td>
<td>1</td>
<td>2.56</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>IIT</td>
<td>42</td>
<td>30</td>
<td>100</td>
<td>9</td>
<td>30</td>
<td>13</td>
<td>43.33</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IIIT</td>
<td>44</td>
<td>40</td>
<td>95</td>
<td>19</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 3: Effect of testosterone on sex ratio among five anuran species.

*Number: O, onset of experiment; Sp., species.
Significant difference from the control in the number of males and females (2 test, $P < 0.05$).
l_1 and 30% in group II_1), gonads were classified as testes due to the presence of testicular cords (Fig. 4F). Such testicular cords were underdeveloped, small, and contained a visibly decreased number of gonial cells. Likewise, in E_2-treated tadpoles, intersex gonads predominated in group II (43.33%) but were not found in groups I_1 and II_1. Only in group I_1, the structure of the Bidder’s organ was visibly altered during metamorphosis (Fig. 4I). No meiotic cells were observed in this organ. The impairment of gonadal development was not observed in individuals reared in the presence of sex steroids starting from Gosner stage 44, i.e. when gonadal sex is established (groups III). Sex ratio was not disrupted visibly (M:F 1 after E_2 treatment and 0.96 after testosterone treatment; P>0.05, Tables 2 and 3). The morphology of such gonads and Bidder’s organ did not deviate from controls (Fig. 4A, D and G). However, no individual from groups III with intersex gonads was found following metamorphosis (Tables 2 and 3).

**Discussion**

Our current study reveals a wide range of effects of sex steroids among several anuran species: developmental retardation, partial sex reversal manifested by hermaphroditic gonads, complete sex reversal (unisexual progeny), and a lack of a detectable effect, depending both on the species and the time of exposure. Our observations allow us to propose a hierarchy of sensitivity among analyzed species of anurans, as described in the following sections.

**Highly sensitive species**

Our study showed that the fire-bellied toads *B. bombina* and *B. variegata* are highly susceptible to sex steroids. Their gonadal development was impaired and the sex was partially reversible through exposure to E_2 and testosterone, as indicated by the increased frequency of intersex and undifferentiated gonads. Our data contradict the results of an early study by Gallien (1974), which suggested that *B. variegata* and *B. orientalis* are refractory to sex steroids. Our previous study showed that the first signs of normal sexual differentiation in *Bombina* appear in male gonads relatively early at Gosner stage 33 when the germ cells translocate from the cortex to the medulla (Piprek et al. 2010). Importantly, it has been previously demonstrated that the effect of hormone depends on the timing of administration (Villalpando & Merchant-Larios 1990, Saidapur et al. 2001, Hogan et al. 2008). Exposure to testosterone or E_2 before sexual differentiation usually causes total sex reversal; exposure beginning during the sexual differentiation results in the formation of ambivalent gonads, whereas exposure after the critical sensitive period of gonad differentiation has no effect (Villalpando & Merchant-Larios 1990). In our study, we obtained a sex distortion in progeny of especially *B. variegata* and also *B. bombina* when exposure to E_2 was started at hatching, indicating high susceptibility of these anurans to sex steroids. These data imply that in previous studies (e.g. Gallien 1974), steroid hormones may have been administered too late to cause any effect.
Our results on *X. laevis* showing the predominance of females among E2-treated *Xenopus* tadpoles are consistent with the previous findings (Wallace et al. 1999). Early studies on *X. laevis* have shown that it was possible to completely reverse male sex by exposure to E2 but that female sex was not reversible by testosterone treatment (Chang & Witschi 1955, Gallien 1956). As *X. laevis* females are heterogametic (ZW), their response to hormone exposure contradicts Witschi’s concept according to which only the male sex can be reversed by sex steroids. It was shown that a complete sex reversal (up to 100% females) was possible only when E2 was added during the undifferentiated stages of gonadal development (Villalpando & Merchant-Larios 1990). When the compound was administered during sexual differentiation of gonads, hermaphroditic gonads are formed, which demonstrates an early commitment of gonads to the female sex. Once the gonadal sex has been established, sex hormones are unable to alter it.

**Less sensitive species**

In hylids, males are heterogametic (XY) and, so far, the studies on the effects of sex steroids on gonadal development in this family have been restricted to *Hyla japonica* and *Pseudacris nigrita* (Takahashi 1958, 1959, Witschi et al. 1958a). Studies on *H. japonica* showed that gonadal sex was partially reversed by E2 and testosterone. E2 caused either retardation of testis development, acceleration of ovary development, small size of the gonad, intersex gonads, or a lack of detectable effects. In *H. japonica*, the sex ratio was skewed toward females among E2-treated tadpoles (Takahashi 1958, 1959). After testosterone exposure, there was a reduction of germ cell number in ovaries and testes, and a few individuals had intersex gonads with germ cells located in both cortex and medulla (Takahashi 1958). Interestingly, in *Pseudacris nigrita* and in the grass frog (*Rana temporaria*), testosterone caused complete sex reversal (Witschi et al. 1958a). The gonadal development of the European tree frog (*H. arborea*) has not been studied previously. In our current study, we did not detect a high rate of complete sex reversed individuals after E2 treatment. However, partial sex reversal was evidenced by the development of intersex gonads. Moreover, we observed degenerating germ cells and a decrease in the number of meiocytes in the ovaries of individuals treated with E2. The better differentiation of the medulla and the absence of the secondary cavity in this gonadal region in *H. arborea* treated with testosterone (Takahashi 1958) were also observed in *H. japonica* treated with testosterone (Takahashi 1958), which suggests taxon-specific similarities regardless of the geographical origin of hylid species. This indicates that sex steroids influence the somatic part of the gonad, which drives sexual differentiation, whereas the germ cells play a minor role in this process.

Among the species belonging to the Bufonidae family, the influence of hormones on gonads has been well...
established only for *Bufo americanus* (Chang 1955). It was shown that these toads can be feminized by E2, but testosterone had no effect on gonadal sex. Gonads in this anuran family are unique due to the presence of Bidder's organ (McDiarmid 1971, Ogielska & Bartmańska 2009). This organ is an ovary-like structure located at the anterior end of both female and male gonads. In *B. viridis* (XY), testosterone caused retardation of Bidder's organ development and the appearance of a cavity that is absent in the normal organ (Vegni Tallur & Padoa 1953). Our studies showed that E2 caused inhibition of gonadal development, and some signs of impaired testicular differentiation, such as the presence of rudiments of testicular cords, were observed. Testosterone led to significant inhibition of *B. viridis* gonadal development; during metamorphosis, these gonads resembled undifferentiated genital ridges. The testosterone sensitivity in *B. viridis* distinguishes this species from *B. americanus*, suggesting more subtle differences in the sensitivity to sex hormones within the family Bufonidae. Interestingly, *B. viridis* individuals with undifferentiated gonads predominated when sex steroids were administered since hatching; however, numerous hermaphroditic gonads were observed when sex steroids, either testosterone or E2, were administered during the sexual differentiation of the gonads, i.e. about Gosner stage 43. This proves that the sex steroid effect depends on the developmental stage at which exposure to sex steroids began.

**Interplay between temporal, structural, and genetic cues in gonad development**

During the period of sexual differentiation, gonads have the potential to develop into intersex organs due to significant structural rearrangements.
process, basal laminae disintegrate and germ cells migrate into the medulla from the cortex in differentiating testis while they stay within the cortex in the ovary (Piprek et al. 2010). At this time of sexual differentiation of gonads, their definite structure is not yet established. Exogenous sex steroids administered at this time may cause ambiguities observable as partial sex reversal. Earlier exposure to hormones influences the molecular pathway of sex determination and thus leads to complete sex reversal. Our present study showed that exposure to sex steroids after establishment of gonad structure has no effect on the gonadal sex. Thus, gonadal structure after sexual differentiation seems insensitive to sex hormones.

The observations of the cell arrangement in the intersex gonads implied that the location of germ cells within the gonad influences their meiotic status. The fate of germ cells, i.e. the germinal sex, is the switch between oogonial or spermatogonial path of differentiation, of germ cells, i.e. the germinal sex, is the switch between oogonial or spermatogonial path of differentiation, which takes place early during gonadal development. Meiosis is induced just after differentiation of the ovary, while in the testis meiosis is suppressed until after metamorphosis or the first year of life (Witschi 1971, Ogieska & Bartmańska 2009, Piprek et al. 2010). We observed that the germ cells located in the cortex of intersex gonads enter meiosis and correspond to preleptotene oocytes in ovaries. Germ cells located in the medulla of intersex gonads never enter meiosis and remain as gonial cells. These results indicate that the environment created by somatic cells determines the fate of germ cells. Somatic cells of the cortex, corresponding to prefollicular cells, probably induce meiosis, whereas somatic cells of the medulla, corresponding to pre-Sertoli cells, inhibit meiosis.

Conclusions
Further studies are required to elucidate the molecular and cellular mechanism of hormone role in gonad differentiation, taking in to account the interspecies differences and the relationship between the type of gonadogenesis and sex determination. Nevertheless, our experiments conducted on five anuran species allowed us to conclude that diverse effects of hormonal exposure result from various processes driving gonadal differentiation and are independent on chromosomal status determining heterogametic state or type of gonadal development. They also revealed important differences in the sensitivity of different species to exogenous hormones. This observation may be useful in identification of factors responsible for the rapid decline of some amphibian species in polluted environments (e.g. Bombina and Xenopus) and may help to explain the relative resistance of other species to environmental perturbation (e.g. Hyla and Bufo).

Materials and Methods

Animals
Clutches of fertilized H. arborea L., 1758, and B. viridis L., 1768, eggs were collected in the wild vicinity of Bielsko-Biała (Pogórze Śląskie, Poland); eggs of B. variegata L., 1758, were collected in Skawce (Beskid Makowski, Poland) and eggs of B. bombina L., 1768, were collected near Miechów (Wyżyna Miechowska, Poland). Larvae of X. laevis Daunin, 1802, were obtained in the laboratory by IVF. Eggs and tadpoles were maintained in 10 l aquaria filled with aerated water under a 12 h light:12 h darkness photoperiod at 19°C. Tadpoles of X. laevis were fed with Seramicron (Sera). Tadpoles of the other species were fed with boiled leaves of dandelion daily ad libitum. Tadpoles of X. laevis were staged according to Nieuwkoop & Faber (1994) and other species were staged according to Gosner (1960). All specimens used in the experiments were acquired according to Polish legal regulations concerning the protection of wild species (Dz. U. nr 33, poz. 289, 2005). We obtained permission from the Polish Ministry of Environment Protection and Forestry and approval from the Local Commission for Ethics in Experiments on Animals. We tested 124 B. bombina tadpoles, 103 B. variegata tadpoles, 181 tadpoles X. laevis, 323 H. arborea tadpoles, and 300 B. viridis tadpoles.

Chemicals
E2 (purity ≥98%) and testosterone (purity ≥98%) were purchased from Sigma–Aldrich. To obtain stock solutions, E2 and testosterone were dissolved in absolute ethanol (purity 99.8%, POCH) to the concentration 2 mg/ml. The stock solutions were maintained in light-resistant container at 4°C and replaced with fresh solutions every 2 weeks. Hormone stock solutions (500 µl) were added to 10 l water to the final concentration of hormones 100 µg/l. Ethanol (500 µl)/10 l was used as a negative control. The concentration of hormones used in the experiment reflects concentrations that caused sex reversal in other anurans (Takahashi 1958, 1959, Villalpando & Merchant-Larios 1999, Hayes 1998).

Sex steroid exposure
Tadpoles of each species were divided into three groups exposed to E2 (groups I, II, and III), three groups to testosterone (groups I, II, and III), and one control group (group IV). The groups treated with sex hormones differed with the onset of sex hormones exposure. The stages of hormonal treatment onset were based on the results of previous studies (Takahashi 1958, 1959, Villalpando & Merchant-Larios 1999, Hayes & Menendez 1999, Piprek et al. 2010). In groups I, the sex steroid treatment was started at the stage just after hatching and continued until metamorphosis. In groups II, the treatment commenced at the period of sexual differentiation of gonads, i.e. at Gosner stage 34 in Bombina sp. and H. arborea, at Gosner stage 42 in B. viridis, and at Nieuwkoop stage 51 in X. laevis. The groups III was treated when the gonads were sexually differentiated, i.e. in Bombina sp. and H. arborea starting from Gosner stage 40, in B. viridis starting from Gosner
stage 44, and in X. laevis starting from Nieuwkoop stage 56. Group IV was the control exposed to the solvent only. The control group was maintained in water with ethanol alone at the concentration of 500 µl/l. All tadpoles were reared in 10 l aquarium at a density of three tadpoles/l water. Water with dissolved hormones was replaced every 3 days. Any dead tadpoles were removed.

**Morphological and statistical analysis**

To describe the gonadal development, animals were anesthetized in MS222 (0.1%) and dissected at six sampling points (Table 1). The gonads together with kidneys were fixed in Bouin’s solution overnight and then dehydrated and embedded in paraplast (Sigma). The 6 µm sections were stained with Debreuille trichrome (Kiernan 1990). Each gonad was classified as testis, ovary, and hermaphrodite or undifferentiated gonad based on the location of germ cells within the organ as previously described (Takahashi 1958, 1959, Villalpando & Coe 1980). The morphological and statistical analyses were based on the location of germ cells within the organ as previously described (Takahashi 1958, 1959, Villalpando & Merchant-Larios 1990). Referring to the variation in the structure along the gonad (El Jamil et al. 2008), we analyzed sections of the entire length of the gonads. Images were taken with a Nikon Eclipse E600 light microscope and processed with Corel Photo-Paint 11. The number of males and females was compared to the control using χ² test. Statistical data were analyzed using Statistica 6 PL Software (Kraków, Poland).

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This research was supported by a grant from the MNiSW (N N303 542938). J Z Kubiak was supported by a grant from ARC. R P Piprek was supported by a grant from FNP.

**Acknowledgements**

The authors are grateful to Dr Malgorzata Kloc for valuable discussions and to Dr M Pabijan for English correction. The authors also thank Peter Koopman for reading the revised version of this work and for his valuable comments.

**References**


www.reproduction-online.org


Takahashi H 1958 Gonadal reaction in the tree frog larvae (Hyla arborea japonica Guenther) to the androgen. Journal of the Faculty of Science Hokkaido University. Series VI Zoology 14 92–99.

Takahashi H 1959 Partial feminization of larval gonads of Hyla arborea japonica Guenther introduced by treatment with estradiol. Journal of the Faculty of Science Hokkaido University. Series VI Zoology 14 210–221.


Received 9 February 2012
First decision 12 March 2012
Revised manuscript received 14 May 2012
Accepted 24 May 2012