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Pubertal sexual development and endpoints for disrupted spermatogenesis in the model *Xenopus tropicalis*

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ABSTRACT

Peripubertal models to determine effects of anti-androgenic endocrine disrupting chemicals are needed. Using the toxicological model species *Xenopus tropicalis*, the aims of the study were to 1) provide data on sexual maturation and 2) characterise effects of short-term exposure to an anti-androgenic model substance. Juvenile (2.5 weeks post metamorphosis old) *X. tropicalis* were exposed to 0, 250, 500 or 1000 μg flutamide/L (nominal) for 2.5 weeks. Upon exposure termination, histology of gonads and Müllerian ducts was characterised in detail. New sperm stages were identified: pale and dark spermatogonial stem cells (SSCs). The testes of control males contained spermatozoa, indicating pubertal onset. The ovaries were immature, and composed of non-follicular and pre-vitellogenic follicular oocytes. The Müllerian ducts were more mature in females than males indicating development/regression in the females and males, respectively. In the 500 μg/L group, the number of dark SSCs per testis area was decreased and the number of secondary spermatogonia was increased. No treatment effects on ovaries or Müllerian ducts were detected. To conclude, our present data provide new knowledge on spermatogenesis, and pubertal onset in *X. tropicalis*. New endpoints for evaluating spermatogenesis are suggested to be added to existing assays used in endocrine and reproductive toxicology.

1. Introduction

The development of the reproductive system is tightly regulated by hormones and is consequently sensitive to exposure to endocrine disrupting chemicals (EDCs). A large number of substances in the environment has been shown to inhibit the action of androgens in the organism by antagonizing the androgen receptor [1]. Exposure to such anti-androgenic EDCs are suspected to contribute the observed adverse reproduction outcomes including reduced sperm quality in humans and wildlife [2]. Animal models for toxicological studies are needed in order to provide evidence for cause-effect relationships with regard to adverse effects of exposure to EDCs during critical phases of development such as puberty.

Testosterone is the main androgen involved in spermatogenesis in higher vertebrates [3]. Androgens are produced and secreted by the interstitial Leydig cells and act on Sertoli cells via binding to the androgen receptor or other transcription factors [4]. In mice, the absence of androgen receptors resulted in inhibited meiosis [5] and in frogs, the absence of androgens resulted in arrested germ cell maturation, and a lack of spermatids and spermatozoa [6]. Many chemicals have demonstrated anti-androgenic properties *in vitro* indicating a potential to interfere with sperm development *in vivo* [7–10]. Exposure during the pubertal period to the androgen receptor antagonist flutamide resulted in decreased sperm motility which caused infertility in adult rats [11]. Research findings in amphibians and mammals indicate that, in addition to androgens, thyroid hormones are involved in testis development and function [12–14].

Histological evaluation of sperm stages is challenging as certain stages are morphologically similar and are only distinguished using methods such as scanning electron microscopy [15]. In the amphibian model species *X. laevis,* the histologically characterized sperm stages are: spermatogonial stem cells (SSCs, in some studies referred to as primary spermatogonia), secondary spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa [16–19]. These stages have been reported also in *X. tropicalis* [18–21] using the criteria by Kalt [16] though detailed morphological criteria to identify them histologically

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have not been characterized. The most detailed histological characterisation of sperm stages in amphibians includes the additional stages; gonocytes, and pale and dark SSCs in *Rana esculenta, Pelophylax lessonae* and *P. ridibundus* [15,22]. To our knowledge, there are no previous reports identifying pale and dark SSCs histologically in the most commonly used amphibian model organisms *X. laevis* and *X. tropicalis*. Previous research has identified the early stage of sperm development as a target for developmental exposure to anti-androgens [19,23]. It is therefore important to characterise dark and pale SSCs in *X. laevis* and *X. tropicalis* to enable detailed investigation into spermatogenesis and the effects of anti-androgens and other substances.

Xenopus frogs (*tropicalis* and *laevis*) have proven useful as toxicological models to investigate endocrine and reproductive toxicity following early life exposure to EDCs [24–27]. In *X. tropicalis*, reproductive development includes the sex determination which occurs at the larval stage, and the sex differentiation (gonadal and brain maturation), and secondary sex characteristics development that starts during the larval stage and continues up to 3 months post metamorphosis (PM) for males and 4.5 months for females, when the animals have reached sexual maturation [28–30]. Mature germ cells (spermatozoa or mature oocytes) in the gonads, oviducts (developed from Müllerian ducts) in females and vas deferens in males are present only after sexual maturation. In addition, secondary sex characteristics, such as a pear-shaped body in female and nuptial pads (breeding "glands") on the forearms of males are also regulated by sex hormones and develop during sexual maturation in *Xenopus* [31]. There is however little information on the onset of specific processes of the sexual development, such as spermatogenesis in *Xenopus*. These questions need to be addressed to further develop *Xenopus* as a toxicological model.

The overall aims of the present study were to increase the understanding of peripubertal sexual development including pubertal onset and to explore potential endpoints for anti-androgenic effects in juvenile *X. tropicalis*. Specifically, the objectives were to: 1) characterize gonadal maturity, spermatogenesis, oogenesis, and Müllerian duct maturity, and 2) characterize apical and detailed histological effects of the anti-androgenic model substance flutamide on sexual maturity in juvenile *X. tropicalis*.

2. Material and method

2.1. Experimental design

To address the first aim, a detailed histological characterisation of gonadal and Müllerian duct maturity was conducted, and pubertal onset was assessed in the control animals. Pubertal onset was assessed by determining the presence of mature germ cells and secondary sex characteristics (nuptial pads or pear-shaped body). If one of these criteria was fulfilled, the animal was classified as having entered puberty. Gonadal maturity was assessed by determining the most mature germ cell present and Müllerian duct maturity was assessed using a scoring system based on histology. The spermatogenesis and oogenesis (distribution germ cell stages in the gonad) were characterized, and gonadal size (cross-section area) was measured and related to the number of germ cells.

To address the second aim, control and anti-androgen treated groups were compared with regard to detailed histological characterisation of gonadal and Müllerian duct maturity as well as spermatogenesis, oogenesis, gonadal size and apical endpoints. The apical endpoints included presence of secondary sex characteristics, hind limb length (as an endpoint for thyroid disruption) and growth (body weight and length).

The age of analysis was based on previous observations regarding sexual maturation in *X. tropicalis*. At four weeks PM, males have not entered puberty, but have an active spermatogenesis and at eight weeks PM they have entered puberty as shown by the presence of mature spermatozoa in the testis [20,21]. However, there is scanty information on gametogenesis, gonadal maturity or effects of EDCs in the period in-between these time points for *X. tropicalis*. The duration of the exposure was based on the OECD test: Fish Short Term Reproduction Assay [32] using juvenile Japanese medaka (*Oryzias latipes*) [33], but with minor changes due to practical reasons.

2.2. Animal husbandry and exposure

Juvenile *X. tropicalis* frogs (obtained from in-lab mating of adult animals originating from Xenopus1, Dexter, MI, USA) at the age of 17.5 ± 4 days PM were exposed for 17 ± 2 days to flutamide (reference standard, CAS: 13311–84–7, Sigma Aldrich) at three nominal concentrations: 0 (Control), 250 (Low), 500 (Mid) and 1000 (High) μg/L under semi-static conditions. Acetone (0.0008 %) was used as a solvent in all tanks including controls. Half of the water and flutamide/acetone solution or acetone alone was exchanged three times per week. To saturate the aquaria with the test compound, the water changing routine was started one week prior the start of the exposure. Two replicate tanks (15 L, Ferplast, Vicenza, Italy) were used for all flutamide treatments, and three for the control group. There were 12–25 animals in each tank (Supplementary table S1). The variation in numbers of juveniles per tank was due to a difference in time to reach metamorphosis which resulted in a variable number of age-matched individuals that could be assigned to an exposure tank at any given time point.

The animals were kept in a 12:12 light:dark cycle (with one hour dawn and one hour dusk). Once a week, before water change, nitrite (standard kit from Sera, Gibbon, Sweden), ammonia/ammonium (standard kit from Sera, Gibbon, Sweden), oxygen saturation, and pH, were recorded in all the tanks, and temperature was monitored daily. The animals were fed Sera vipan baby (Sera, Heinsberg, Germany), and Energy food (Sera, Heinsberg, Germany) three times per day in weekdays and once a day with double amount on weekends. This study was approved by Uppsala Ethics Committee for Animal Care and Use (5.8.18–09239/2018) and Uppsala University and carried out in accordance with relevant guidelines and regulations including the ARRIVE guidelines.

2.3. Sampling and apical endpoints

Upon discontinuation of the exposure, at five weeks PM, the frogs were sacrificed by decapitation after first being anaesthetised in 0.3 % buffered Tricaine (Sigma-Aldrich, Saint-Louis, USA). The hind limb length, snout-vent length (SVL) and body weight were measured. Any presence of nuptial pads and a pear-like body shape was noted. The gonad-kidney complex (including kidneys, gonads and Müllerian ducts) was fixed in 4 % buffered formaldehyde for histological processing and analysis of gonadal and Müllerian duct histology. Brain, thyroid and the rest of the body (without intestines) were also sampled during dissection, but for purposes beyond the present study. During the dissection, the evaluator was aware of which exposure the animals had been subjected to.

2.4. Histological processing

The gonad-kidney complexes were dehydrated in increasing concentrations of ethanol and embedded in hydroxyethyl methacrylate after being infiltrated overnight (Technovit 7100, KULZER GmbH, Germany). Transversal sections were taken at three levels with a distance of 300 μm in-between, starting from the anterior part of the gonads from 26 Controls, 10 Low, 11 Mid and 26 High animals. One Section (2 μm thick) per individual, from the centre of the gonad, was stained with haematoxylin-eosin, the other sections were stained with toluidine blue. The sample size was based on previous studies on histological effects on gonads [23].

2.5. Histological analysis

The histological slides were scanned with a histological scanner (NanoZoomer 2.0-H, Hamamatsu) and evaluated using NDP.view (Hamamatsu Photonics K.K, version 2.7.52, 2019). Gonadal distribution of germ cell stages and gonadal size (cross-section area) were assessed in one haematoxylin-eosin stained section from the center of the gonads for each individual. Determination of the most mature germ cell present was assessed using all three sections per individual. For each individual, both the left and right hand side gonad were evaluated and a mean value of the data from the two was used for statistical analysis. All histological analyses were performed by one person using coded slides.

2.5.1. Testis histology and spermatogenesis

The sperm stages that were analysed included gonocytes, pale SSCs, dark SSCs, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa using a combination of morphological criteria presented in Table 1 [15–17,22]. The number of cells per sperm stage was determined in 15 Controls, 7 Low, 6 Mid and 6 High, and the most mature germ cell stage was noted for each individual. The testis cross-section area was measured with ImageJ (National Institute of Health, Bethesda, MD, USA) and related to number of germ cells.

2.5.2. Ovary histology and oogenesis

In 12 Control, 3 Low, 4 Mid and 20 High females, the number of oocytes was determined, and the most mature germ cell present noted. The following non-follicular oocyte types were counted: premeiotic oogonia and oocytes in very early meiotic prophase i.e. primary oogonia, secondary oogonia, preleptotene oocytes, leptotene oocytes, pachytene oocytes and early diplotene oocytes. The follicular oocyte types counted were: stage I, II, III, IV, V and VI oocytes [34]. The oocytes were then combined into the two categories and a ratio of follicular: non-follicular oocytes was calculated. Ovary cross-section area was measured with ImageJ (National Institute of Health, Bethesda, MD, USA) and related to the number of oocytes.

Table 1
Summary of histological characteristics and functional features for sperm cell stages in amphibians. These morphological criteria were used for the identification of sperm cell stages in juvenile Xenopus tropicalis.

Sperm stage	Histological characteristics	Functional feature	Species[a] (reference)
Gonocytes	Large, single cells. Occupying the major part/space of the sex cords. One or two nucleoli.	Only present during pre-spermatogenesis. Differentiate into pale SSCs.	*Pelophylax Lessonae* ([15]) *P. ridibundus* ([15])
Pale SSCs	Large, single cells. Slightly irregular shape, with light pink cytoplasm and homogenous chromatin.	Form the stem cell reserve. Differentiate into dark SSCs or self-renew.	*P. lessonae* ([15]) *P. ridibundus* ([15]) *Rana esculenta* ([22])
Dark SSCs	Round cells. Similar appearance as pale SSCs but smaller and with a darker cytoplasm and more heterochromatin. Present individually or in clusters of two.	Differentiate into secondary spermatogonia.	*P. lessonae* ([15]) *P. ridibundus* ([15]) *R. esculenta* ([22])
Secondary spermatogonia	Clearly organized into spermatocysts. The nucleus is irregularly shaped either round or slightly elongated, with visible nucleoli and chromatin patches. Undergo mitosis, hence their size is gradually decreased, and the cell shape is altered from elongated to more round.	Proliferate and differentiate into primary spermatocytes	*Xenopus laevis* ([16,17]) *P. lessonae* ([15]) *P. ridibundus* ([15])
Primary spermatocytes	Chromosome appearance typical to the phase of meiosis (prophase, metaphase, anaphase or telophase). Cells in prophase are round with a nucleus that is large in relation to the cytoplasm, containing loosely packed chromatin. Cells in later meiotic phases are classified according to the stage of chromosomal division.	Undergo the first meiosis.	*X. laevis* ([16]) *P. lessonae* ([15]) *P. ridibundus* ([15])
Secondary spermatocytes	Round, smaller than primary spermatocytes, with a small, round, condensed nucleus.	Undergo the second meiosis	*X. laevis* ([16]) *P. lessonae* ([15]) *P. ridibundus* ([15])
Spermatids	Either completely round or slightly elongated. Condensed nucleus and dissolved cytoplasm. Cells connected via a spider net-like structure.	Differentiate into spermatozoa	*X. laevis* ([16]) *X. tropicalis* ([18]) *P. lessonae* ([15]) *P. ridibundus* ([15])
Spermatozoa	Elongated cells, completely lacking cytoplasm. Either in nests, with heads pointing towards the lumen or released and free in the lumen.	Mature sperm	*X. laevis* ([16]) *X. tropicalis* ([18]) *P. lessonae* ([15]) *P. ridibundus* ([15])

SSCs: spermatogonial stem cells.
[a] Species for which histological characteristics are presented for the specific sperm stages.

2.5.3. Müllerian duct maturity

Müllerian duct maturity was evaluated on a toluidine stained slide containing sections from the middle of the gonad in males (15 Control, 7 Low, 6 Mid and 6 High) and females (12 Controls, 3 Low, 4 Mid and 20 High). The Müllerian ducts were assigned maturity scores from 1 to 5 as defined in Jansson et al., (2016). Score 1 was assigned to the earliest stage ducts characterised by a small protrusion of loosely packed connective tissue from the kidney. The criteria for score 5 ducts were: a clear tubular structure with a cavity, directly adjacent to the kidney. The criteria for scores 2–4 include increasing formation of a tubular structure.

2.6. Statistical analysis

The data from all replicate tanks were visually examined, to estimate the variability. If no apparent difference between the replicates, the data were pooled according to level of exposure. Linear regression analyses were conducted to evaluate the relationship between gonadal area and the number of germ cell in each stage in control males and females. Müllerian duct maturity in control males and females were compared using Mann Whitney test.

Mortality in the treatment groups was compared to the control group using Fisher's exact test. Generalized linear models were used to test for treatment effects and different distributions and link functions were selected in order to better fit the data. A Gaussian family of distribution with identity link function was used for normally distributed data (body weight, SVL, hind limb length and germ cell counts/area) and gamma family of distribution with log link function was used for non-normal positive continuous data (gonadal area and Müllerian duct maturity). The quality of the models was assessed by checking the distribution of the residuals. Multiple comparisons of treatments to control were performed and p-values were adjusted using the Holm-Bonferroni method. Gonadal maturity (the most mature germ cell present) was analysed with the Chi-square test.

Generalized linear models were conducted in R version 4.0.2 (R Core Team, Vienna, Austria) and R Studio version 1.3.1093 (RStudio Team, Boston, MA, US) where the package DHARMa was used for model diagnostics and emmeans was used for multiple comparisons. Fisher's exact test, Chi-square test, Mann Whitney test and linear regression, were performed in GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Data were considered statistically significant if $p \leq 0.05$.

3. Results

3.1. Husbandry conditions, and apical endpoints

There was no significant difference in mortality between controls and treatments (3/56, 4/36, 4/44 and 1/37 individuals for Control, Low, Mid and High respectively) and no other signs of treatment related health impairment. No treatment effects on body weight, SVL or hind limb length were found (Supplementary table S3) with the exception that the High males had increased SVL compared to Control males ($p = 0.0213$). No secondary sex characteristics were observed in the males nor females. Information on husbandry conditions is found in Supplementary table S2.

3.2. Testicular histology and spermatogenesis

The testes mainly consisted of seminiferous cords and a few seminiferous tubuli characterised by a lumen (Fig. 1A). All animals had established the dark SSCs in the testis, and no gonocytes (precursor cells to SSCs) were therefore identified. The pale and dark SSCs (Fig. 1B), and spermatozoa (Fig. 1G) were the germ cell stages easiest to distinguish as their morphology was completely different compared with the other stages. The SSCs appeared as large single cells inside cysts and the spermatozoa were mainly present as detached from the germinal epithelium, free in the lumen. Primary spermatocytes (Fig. 1D) were morphologically similar to later stages of secondary spermatogonia (Fig. 1C), except with regard to the appearance of the nuclei and the packing of chromatin. In secondary spermatogonia, a nucleolus was not always visible and, therefore, chromatin patches, packing of the chromatin and the shape of the nucleus were more reliable morphological features. Secondary spermatocytes (Fig. 1E) were only observed in one nest in one animal and they did not show the typical features of second

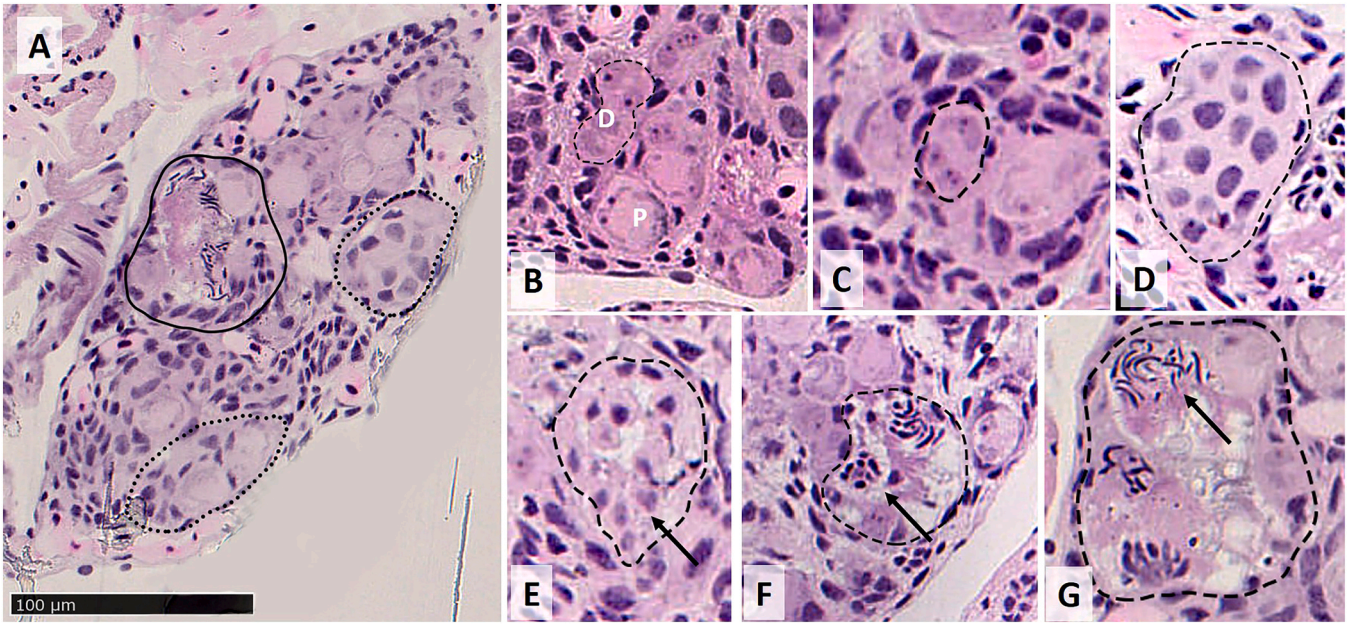

Fig. 1. Histomicrographs of testes from juvenile *Xenopus tropicalis*, five weeks post metamorphosis, showing A) seminiferous chords (dotted line) and seminiferous tubuli containing a lumen (solid line), B) spermatogonial stem cells (SSCs); pale SSCs (P) and dark SSCs (D), C) nests with proliferating secondary spermatogonia, D) nest with primary spermatocytes, E) secondary spermatocytes starting to differentiate into spermatids (arrow), F) spermatids (arrow). G) Fully mature spermatozoa (arrow). Sections are stained with haematoxylin-eosin. Dashed lines encircle germ cell nests.

meiosis. Spermatids (Fig. 1F), were also rarely observed but were easily distinguished from the secondary spermatocytes due to their almost complete lack of cytoplasm, and very round nucleus.

In the Control males, the frequencies of testicular maturity stages, as determined by the most mature germ cell stage observed, were: 6/15 with fully mature spermatozoa in the seminiferous tubule lumen, 4/15 with primary spermatocytes, 4/15 with secondary spermatogonia and 1/15 with dark SSCs.

The total number of SSCs per testis cross-section area was not correlated with the cross-section area in the Controls. However, when the SSCs group was split into the two subtypes, it was found that the number of dark SSCs was positively correlated to the testis cross-section area ($p = 0.0443$, $r^2 = 0.28$, linear regression), whereas the number of pale SSCs was not (Supplementary Fig. S3). The numbers of secondary spermatogonia and primary spermatocytes did not show any significant correlation to the testis cross-section area either. No correlation analyses were performed on the other germ cells (secondary spermatocytes, spermatids and spermatozoa), as the frequencies of testis showing presence of them were too low.

In the Mid group, there was a significant decrease in the number of dark SSCs per testis area ($p = 0.0142$) and a significant increase in the number of secondary spermatogonia ($p = 0.0040$) compared to Control (Table 2). No other significant difference between treatments and Control with regard to number of germ cell stages in relation to area was detected. No differences between the Control and treatment groups with regard to testis area (Table 2) or testis maturity (Fig. 2) were found.

3.3. Ovarian histology and oogenesis

All Control ovaries consisted of non-follicular and follicular previtellogenic stage I or II oocytes (Supplementary Fig. S1). No mature stage VI oocytes were detected. There was a positive correlation between ovary cross-section area and total number of oocytes ($r^2 = 0.93$, $p < 0.0001$). This was driven by the follicular oocytes as the area positively correlated to number of follicular oocytes ($r^2 = 0.88$, $p < 0.0001$, linear regression), but not to the number of non-follicular oocytes (Supplementary Fig. S4). In all females but three in the High group, follicular oocytes were observed. No significant difference between Control and treatments was detected with regard to ovary maturity, ovary cross-section area, the number of non-follicular, follicular or total oocytes, or the ratio follicular:non-follicular oocytes (Table 3).

3.4. Müllerian duct maturity

In 6/15 Control males, a Müllerian duct score of 2 for both right and left duct was determined and in 2/15 individuals, both ducts were scored 1 were. In several males, the two ducts were given different maturity scores. In 5/15 males one duct was assigned score 2 and the other score 1, and in 1/15 males the two ducts were assigned score 2 and score 3 (Supplementary Fig. S2). One male could not be evaluated as the lateral parts of the kidneys including the Müllerian ducts were missing from the section.

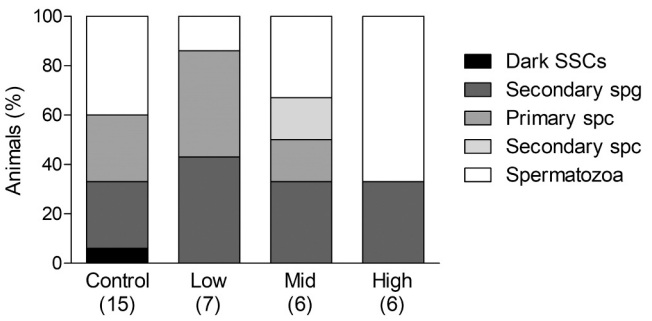

Fig. 2. Testis maturity as determined by the most mature germ cell stage observed per male *Xenopus tropicalis* after short-term peripubertal exposure to 0, (Control), 250 (Low), 500 (Mid) or 1000 (High) μg flutamide/L. SSCs: spermatogonial stem cells, spg: spermatogonia, spc: spermatocytes. Number of individuals (n) is shown within the parenthesis.

In 4/12 Control females, a score of 2 was determined for both ducts. In 4/12 females both ducts were given the score 3 and in 1/12 females, the score 5 was assigned to both ducts. In 2/12 females, the right duct was given the score 3 and the left duct was assigned score 2. In 1/12 females, the right duct was given score 2 and the left duct the score 1 (Supplementary Fig. S2). The mean Müllerian duct maturity score in the Control group was significantly higher in females than in males ($p = 0.0024$, Mann Whitney test) (Table 4). No difference in Müllerian duct maturity between treatments in either of the sexes was found (Table 4).

4. Discussion

In the present study, we aimed to characterise sexual development including gametogenesis in peripubertal *X. tropicalis* and potential effects of anti-androgenic EDCs on these processes. Using detailed histological analysis of the testes we identified dark and pale SSCs, two sperm stages that, to our knowledge, were previously not described in Xenopus. We found that male *X. tropicalis* can reach sexual maturity in 5 weeks PM. The number of dark SSCs and secondary spermatogonia were altered in flutamide exposed males (500 μg/L) suggesting that processes regulating these stages in spermatogenesis may be targeted by exposure to anti-androgens during pubertal development.

By the histological identification of dark and pale SSCs in *X. tropicalis*, we found all sperm stages that were previously described in other amphibian species i.e. *X. laevis*, *P. lessonae* and *P. ridibundus* [15–17]. Haczkiewicz et al. [15] concluded that the sperm stages described in anurans can be translated to the mammalian sperm stages i.e. A spermatogonia, B spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. Accordingly, A_{dark} and

Table 2
Detailed histological analysis of testes in juvenile *Xenopus tropicalis* five weeks post metamorphosis after exposure for 17 ± 2 days to 0 (Control), 250 (Low), 500 (Mid) or 1000 (High) μg flutamide/L. Data are presented as mean (SD) and represent both gonads for each individual.

	Control (n = 15)	Low (n = 7)	Mid (n = 6)	High (n = 6)
Testis area (mm^2)	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)
Total SSCs (no./mm^2)	914.0 (411.6)	1052.4 (437.9)	667.2 (199.0)	928.4 (346.6)
Pale SSCs (no./mm^2)	432.6 (285.5)	707.3 (383.2)	453.2 (169.5)	624.6 (308.1)
Dark SSCs (no./mm^2)	481.4 (222.3)	345.0 (244.8)	214.0 (108.0)*	303.8 (92.4)
Secondary spermatogonia (no./mm^2)	335.1 (311.8)	379.2 (215.4)	870.0 (633.2)**	476.9 (172.8)
Primary spermatocytes (no./mm^2)	124.2 (169.1)	99.6 (137.2)	96.8 (182.2)	283.6 (284.0)
Secondary spermatocytes (no./mm^2)	23.7 (72.6)	0.0 (0.0)	39.6 (97.1)	26.7 (65.5)
Spermatids (no./mm^2)	1.4 (5.2)	0.0 (0.0)	0.0 (0.0)	10.5 (20.3)
Lumen with spermatozoa (no./mm^2)	9.0 (16.9)	6.7 (17.2)	2.6 (6.5)	40.3 (48.5)

SSC: spermatogonial stem cell. *Statistically significant from control ($p < 0.05$). **Statistically different from control ($p < 0.01$). Generalized linear model (negative binomial distribution, log link) with Holm-Bonferroni as post hoc test).

Table 3
Detailed histological analysis of ovaries in juvenile *Xenopus tropicalis* five weeks post metamorphosis after exposure for 17 ± 2 days to 0 (Control), 250 (Low), 500 (Mid) or 1000 (High) μg flutamide/L. Data are presented as mean (SD) and represent both gonads for each individual.

	Control (n = 12)	Low (n = 3)	Mid (n = 4)	High (n = 20)
Ovary area (mm^2)	0.10 (0.08)	0.05 (0.04)	0.07 (0.02)	0.09 (0.07)
Total oocytes (no./mm^2)	983.3 (481.0)	1170.7 (601.9)	989.1 (128.9)	1232.0 (659.2)
Non-follicular oocytes[a] (no./mm^2)	495.2 (363.4)	773.1 (519.0)	459.5 (263.1)	741.0 (565.5)
Follicular oocytes[b] (no./mm^2)	488.1 (224.9)	397.6 (315.0)	621.2 (516.9)	491.0 (481.0)
Follicular:non-follicular oocytes	1.65 (1.22)	0.92 (0.75)	0.72 (0.27)	1.09 (0.83)

[a] Premeiotic oogonia and oocytes in very early meiotic prophase i.e. primary oogonia, secondary oogonia, preleptotene oocytes, leptotene oocytes, pachytene oocytes and early diplotene oocytes.
[b] Previtellogenic, follicular stage I and II oocytes.

Table 4
Histological analysis of Müllerian duct maturity[a] in juvenile *Xenopus tropicalis* five weeks post metamorphosis after exposure for 17 ± 2 days to 0 (control), 250 (low), 500 (mid) or 1000 (high) μg flutamide/L. Data are presented as mean (SD) and represent both ducts for each individual.

Control		Low		Mid		High	
Male (n = 14)	Female (n = 12)	Male (n = 6)	Female (n = 3)	Male (n = 6)	Female (n = 4)	Male (n = 6)	Female (n = 20)
1.7 (0.4)	2.6 (0.9)**	1.8 (0.6)	2.7 (0.6)	1.9 (0.2)	2.0 (0.3)	2.0 (0.5)	2.6 (0.9)

[a] Maturation score based on criteria by Jansson et al., 2016. "1 - a small rounded bulge of irregularly packed mesenchymal cells at the lateral side of the kidney, 2– a small bud protruding from the kidney, 3 – a distinct structure attached to the lateral side of the kidney, 4 – a distinct tubular structure without a cavity, and 5 – a distinct tubular structure with a cavity lined by elongated epithelial cells". * *Significantly different from Control males ($p < 0.01$), Mann Whitney test.

A_{pale} spermatogonia (monkeys and humans) or A_{single} (rodents) are equivalent to SSCs in amphibians, and B spermatogonia (monkeys and humans) and A_{paired}, $A_{aligned}$ and B spermatogonia (rodents) equivalent to secondary spermatogonia in amphibians. The different types of A spermatogonia are scarcely found and are difficult to distinguish in mammals, hence information about anti-androgenic effects on early spermatogenesis in mammals is very limited [35,36]. The *X. tropicalis* model may therefore be a valuable animal model for studies on the spermatogenesis, in particular the early stages.

The presence of mature sperm suggests that the male frogs had entered puberty. We have previously detected mature spermatozoa at eight weeks PM [21]. The present results further specify the onset of puberty for male *X. tropicalis* to five weeks PM. The presence of mature spermatozoa implies that intratesticular testosterone levels were sufficient to induce spermatogenesis. However, no nuptial pads were noticed, indicating that the concentration of circulating testosterone was likely still too low at this age for induction of their development. The female *X. tropicalis* had not entered puberty which was expected [28]. At five weeks PM the ovaries were similar in composition as at four and eight weeks PM [20,21], with no vitellogenic or mature oocytes observed. The timing of regression and sexual dimorphism of Müllerian ducts in four weeks PM *X. tropicalis* has been unclear [20,29]. In the present study, the ducts in males were less mature compared to those in females. This indicates that the time around four-five weeks PM might be the start of the development of Müllerian duct sexual dimorphism, resulting from Müllerian duct growth in females and/or regression in males. In the present study, gonadal histology was the earliest and most unambiguous indicator of pubertal onset.

The dark SSCs was the only germ cell stage for which the number of cells was positively correlated to testis area, indicating that testis growth was associated with either increased differentiation of pale SSCs into dark SSCs or proliferation of dark SSCs. Regardless of which, the present results imply that testis growth during the start of puberty is driven by activation of spermatogenesis as defined as the formation of dark SSCs which is the germ cell stage entering the spermatogenic cycle in juvenile male *X. tropicalis*.

The increased number of secondary spermatogonia, and decreased number of dark SSCs observed in the testes of the males of the Mid group, suggest that short-term flutamide exposure during the prepubertal period interfered with the pre-meiotic phase of spermatogenesis. Follicle stimulating hormone (FSH) promotes SSC differentiation via activine A and follistatin, whereas androgens have an inhibitory effect on SSC differentiation and proliferation by exerting negative feedback on the hypothalamic-gonadal-axis in mammals [37,38]. Inhibition of the negative feedback in flutamide exposed frogs, may have stimulated increased FSH secretion, thereby causing increased differentiation of dark SSCs into secondary spermatogonia. Developmental exposure to flutamide has been shown to increase the total number of spermatogonia in amphibians, and to cause effects on spermatogenesis that persist into adulthood [19]. The resolution of the analysis, however, was not enough to establish possible effects on the respective SSCs. Anti-androgenic effects on dark SSC (or the equivalent stage) differentiation have, to our knowledge, not been reported previously in any vertebrate species. The variation in sample size for the histological analyses was a result of some mistakes in the preliminary sexing during dissection and not knowing the definite sex of the frogs until after the histological analysis of the gonads, however, this was not considered to influence the outcome of the analysis. The present study therefore contributes to the general knowledge on potential targets for anti-androgenic EDCs in the spermatogenesis.

In standardized test guidelines for chemical testing, histology based endpoints for adverse effects on spermatogenesis are not adequately specified. The standard tests the Larval Amphibian Growth and Development Assay (LAGDA) and the Peripubertal Male Rats Assay [25,39] include analysis of general testicular histopathology in juveniles, based on severity grading of general pathology. Hence, there is a risk in overlooking small changes in germ cell populations such as SSCs [40]. To increase the sensitivity and resolution of reproductive toxicity assays such as LAGDA to detect potential anti-androgenic/estrogenic effects of chemicals, the number of dark SSCs (or the equivalent) could be included as a fully quantitative and objective endpoint. Such refinement of existing test guidelines would contribute to the principles of the 3Rs (replace, reduce, refine).

The lack of treatment effects on the maturation of the ovaries and Müllerian ducts suggests that these processes were not susceptible to impact of short-term exposure conditions during the early juvenile phase. Androgens are important in oocyte maturation, but mainly at the later follicular stages [41], which might explain the lack of treatment effects on these developmental processes. It cannot however, be ruled out that effects can be seen later in life and five weeks PM may not be an

optimal age to evaluate anti-androgenic effects on the ovary maturation.

The increased SVL in the High males was unexpected and might suggest a stimulatory effect on body growth in juvenile frogs. In juvenile fish, flutamide exposure did not affect the body length [33,42,43]. There is limited information available on the potential mechanism of anti-androgens to affect juvenile body length in male amphibians. This observation is therefore currently not understood.

5. Conclusion

Two new sperm stages, dark and pale SSCs, were identified histologically in *X. tropicalis*. To our knowledge, these sperm stages have previously not been described in *Xenopus*. Additionally, we further specified pubertal onset in males to occur between four and five weeks PM. The number of dark SSCs and secondary spermatogonia were altered in flutamide exposed males suggesting that these stages in spermatogenesis may be targeted by exposure to anti-androgens during pubertal development. In conclusion, the present study contributes with new knowledge on spermatogenesis and sexual development of *X. tropicalis*. We furthermore suggest counts of dark SSCs and secondary spermatogonia as quantitative, objective endpoints for disrupted spermatogenesis to be included in existing test guidelines for EDCs.

CRediT authorship contribution statement

S.S: Conceptualization, Methodology, Investigation, Formal analysis, Data interpretation, Writing – original draft, Writing – review & editing, Visualization. M.R: Formal analysis, Data interpretation, Writing – review & editing. V.B: Data interpretation, Writing – review & editing. D. M: Technical assistance, Data interpretation, Writing – review & editing. O.K: Data interpretation, Writing – review & editing. C.B: Conceptualization, Methodology, Data interpretation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2023.108435.

References

[1] D.C. Luccio-Camelo, G.S. Prins, Disruption of androgen receptor signaling in males by environmental chemicals, J. Steroid Biochem. Mol. Biol. 127 (2011) 74–82, https://doi.org/10.1016/j.jsbmb.2011.04.004.

[2] V.L. Marlatt, S. Bayen, D. Castaneda-Cortès, G. Delbès, P. Grigorova, V.S. Langlois, C.J. Martyniuk, C.D. Metcalfe, L. Parent, A. Rwigemera, P. Thomson, G. Van Der Kraak, Impacts of endocrine disrupting chemicals on reproduction in wildlife and humans, Environ. Res. 208 (2022), https://doi.org/10.1016/j.envres.2021.112584.

[3] J. Singh, D.J. Handelsman, The effects of recombinant FSH on testosterone-induced spermatogenesis in gonadotrophin-deficient (hpg) mice, J. Androl. 17 (1996) 382–393, https://doi.org/10.1002/j.1939-4640.1996.tb01804.x.

[4] W.H. Walker, Molecular mechanisms of testosterone action in spermatogenesis, Steroids 74 (2009) 602–607, https://doi.org/10.1016/J.STEROIDS.2008.11.017.

[5] S. Yeh, M.Y. Tsai, Q. Xu, X.M. Mu, H. Lardy, K.E. Huang, H. Lin, S. Der Yeh, S. Altuwaijri, X. Zhou, L. Xing, B.F. Boyce, M.C. Hung, S. Zhang, L. Gan, C. Chang, Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13498–13503, https://doi.org/10.1073/pnas.212474399.

[6] M. Ogielska, J. Bartmańska, Spermatogenesis and male reproductive system in Amphibia-Anura, Reprod. Amphib. (2009) 34–99.

[7] S. Aït-Aïssa, S. Laskowski, N. Laville, J.M. Porcher, F. Brion, Anti-androgenic activities of environmental pesticides in the MDA-kb2 reporter cell line, Toxicol. Vitro 24 (2010) 1979–1985, https://doi.org/10.1016/j.tiv.2010.08.014.

[8] H. Fang, W. Tong, W.S. Branham, C.L. Moland, S.L. Dial, H. Hong, Q. Xie, R. Perkins, W. Owens, D.M. Sheehan, Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor, Chem. Res. Toxicol. 16 (2003) 1338–1358, https://doi.org/10.1021/tx030011g.

[9] H. Kojima, E. Katsura, S. Takeuchi, K. Niiyama, K. Kobayashi, Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells, Environ. Health Perspect. 112 (2004) 524–531, https://doi.org/10.1289/ehp.6649.

[10] F. Orton, E. Rosivatz, M. Scholze, A. Kortenkamp, Widely used pesticides with previously unknown endocrine activity revealed as in vitro antiandrogens, Environ. Health Perspect. 119 (2011) 794–800, https://doi.org/10.1289/ehp.1002895.

[11] J.E. Perobelli, T.R. Alves, F.C. de Toledo, C.D.B. Fernandez, J.A. Anselmo-Franci, G.R. Klinefelter, W.D.G. Kempinas, Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty, Reprod. Toxicol. 33 (2012) 308–315, https://doi.org/10.1016/J.REPROTOX.2011.12.011.

[12] T.B. Hayes, Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms, J. Exp. Zool. 281 (1998) 373–399, https://doi.org/10.1002/(SICI)1097-010X(19980801)281:5<373::AID-JEZ4>3.0.CO;2-L.

[13] D.R. Holsberger, S.E. Kiesewetter, P.S. Cooke, Regulation of neonatal sertoli cell development by thyroid hormone receptor α1, Biol. Reprod. 73 (2005) 396–403, https://doi.org/10.1095/biolreprod.105.041426.

[14] S.M.L.C. Mendis-Handagama, H.B.S. Ariyaratne, Leydig cells, thyroid hormones and steroidogenesis, Indian J. Exp. Biol. 43 (2005) 939–962.

[15] K. Haczkiewicz, B. Rozenblut-Kościsty, M. Ogielska, Prespermatogenesis and early spermatogenesis in frogs, Zoology 122 (2017) 63–79, https://doi.org/10.1016/j.zool.2017.01.003.

[16] M.R. Kalt, Morphology and kinetics of spermatogenesisin Xenopus laevis, J. Exp. Zool. 195 (1976) 393–407, https://doi.org/10.1002/jez.1401950306.

[17] K. Takamune, T. Kawasaki, S. Ukon, The first and the second mitotic phases of spermatogonial stage in Xenopus laevis: secondary spermatogonia which have differentiated after completion of the first mitotic phase acquire an ability of mitosis to meiosis conversion, Zool. Sci. 18 (2001) 577–583, https://doi.org/10.2108/zsj.18.577.

[18] I. Gyllenhammar, L. Holm, R. Eklund, C. Berg, Reproductive toxicity in Xenopus tropicalis after developmental exposure to environmental concentrations of ethynylestradiol, Aquat. Toxicol. 91 (2009) 171–178, https://doi.org/10.1016/j.aquatox.2008.06.019.

[19] F. Orton, M. Säfholm, E. Jansson, Y. Carlsson, A. Eriksson, J. Fick, T. Uren Webster, T. McMillan, M. Leishman, B. Verbruggen, T. Economou, C.R. Tyler, C. Berg, Exposure to an anti-androgenic herbicide negatively impacts reproductive physiology and fertility in Xenopus tropicalis, Sci. Rep. 8 (2018) 1–15, https://doi.org/10.1038/s41598-018-27161-2.

[20] M. Säfholm, E. Jansson, J. Fick, C. Berg, Molecular and histological endpoints for developmental reproductive toxicity in Xenopus tropicalis: levonorgestrel perturbs anti-Müllerian hormone and progesterone receptor expression, Comp. Biochem. Physiol. Part - C. Toxicol. Pharm. 181–182 (2016) 9–18, https://doi.org/10.1016/J.CBPC.2015.12.001.

[21] S. Svanholm, M. Säfholm, N. Brande-Lavridsen, E. Larsson, C. Berg, Developmental reproductive toxicity and endocrine activity of propiconazole in the Xenopus tropicalis model, Sci. Total Environ. 753 (2021), 141940, https://doi.org/10.1016/j.scitotenv.2020.141940.

[22] M. Ogielska, J. Bartmańska, Development of testes and differentiation of germ cells in water frogs of the Rana esculenta - complex (Amphibia, Anura), Amphib. Reptil 20 (1999) 251–263, https://doi.org/10.1163/156853899507059.

[23] O. Karlsson, S. Svanholm, A. Eriksson, J. Chidiac, J. Eriksson, F. Jernerén, C. Berg, Pesticide-induced multigenerational effects on amphibian reproduction and metabolism, Sci. Total Environ. 775 (2021), 145771, https://doi.org/10.1016/j.scitotenv.2021.145771.

[24] C. Berg, The Xenopus tropicalis model for studies of developmental and reproductive toxicity, in: Methods Mol. Biol., Humana Press Inc., 2019, pp. 173–186, https://doi.org/10.1007/978-1-4939-9182-2_12.

[25] OECD, OECD Guidline for the testing of chemicals 241: The Larval Amphibian Growth and Development Assay (LAGDA), (2015) 1–38.

[26] OECD, OECD Guidline for the testing of chemicals 231: Amphibian Metamorphosis Assay, OECD Guidel. Test. Chem. (2009). https://doi.org/10.1787/9789264076242-en.
[27] W. Kloas, I. Lutz, R. Einspanier, Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo, Sci. Total Environ. 225 (1999) 59–68, https://doi.org/10.1016/S0048-9697(99)80017-5.
[28] N. Hirsch, L.B. Zimmerman, R.M. Grainger, Xenopus, the next generation: X. tropicalis genetics and genomics, Dev. Dyn. 225 (2002) 422–433, https://doi.org/10.1002/dvdy.10178.
[29] E. Jansson, A. Mattsson, J. Goldstone, C. Berg, Sex-dependent expression of anti-Müllerian hormone (amh) and amh receptor 2 during sex organ differentiation and characterization of the Müllerian duct development in Xenopus tropicalis, Gen. Comp. Endocrinol. 229 (2016) 132–144, https://doi.org/10.1016/j.ygcen.2016.03.018.
[30] M. Takase, T. Iguchi, Molecular cloning of two isoforms of Xenopus (Silurana) tropicalis estrogen receptor mRNA and their expression during development, Biochim. Biophys. Acta - Gene Struct. Expr. 1769 (2007) 172–181, https://doi.org/10.1016/j.bbaexp.2007.01.011.
[31] D.M. Sever, N.L. Staub, Hormones, Sex Accessory Structures, and Secondary Sexual Characteristics in Amphibians, in: Horm. Reprod. Vertebr. - Vol. 2, 2011: pp. 83–98. https://doi.org/10.1016/B978–0-12–374931-4.10005–7.
[32] OECD, OECD Guidline for the testing of chemicals 229: Fish Short Term Reproduction Assay, (2012) 8. ⟨http://www.oecd.org/termsandconditions/.%0Ahttps://www.oecd-ilibrary.org/docserver/9789264071087-en.pdf?expires=1564573709&id=id&accname=guest&checksum=F2F4F69F7186532A48B300C9AA379185⟩ (accessed June 30, 2022).
[33] A. Nakamura, H. Takanobu, I. Tamura, M. Yamamuro, T. Iguchi, N. Tatarazako, Verification of responses of Japanese medaka (Oryzias latipes) to anti-androgens, vinclozolin and flutamide, in short-term assays, J. Appl. Toxicol. 34 (2014) 545–553, https://doi.org/10.1002/jat.2934.
[34] P. Hausen, M. Riebesell, The Early Development of Xenopus Laevis: An Atlas of the Histology, Springer,, Berlin, Germany, 1991, https://doi.org/10.1086/417692.
[35] J.M. Oatley, R.L. Brinster, Spermatogonial stem cells, in: Methods Enzymol, Academic Press Inc, 2006, pp. 259–282, https://doi.org/10.1016/S0076-6879(06)19011-4.
[36] S.R. Chen, Y.X. Liu, Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling, Reproduction 149 (2015) R159–R167, https://doi.org/10.1530/REP-14-0481.
[37] T. Meehan, S. Schlatt, M.K. O'Bryan, D.M. De Kretser, K.L. Loveland, Regulation of germ cell and sertoli cell development by activin, follistatin, and FSH, Dev. Biol. 220 (2000) 225–237, https://doi.org/10.1006/DBIO.2000.9625.
[38] N. Sofikitis, N. Giotitsas, P. Tsounapi, D. Baltogiannis, D. Giannakis, N. Pardalidis, Hormonal regulation of spermatogenesis and spermiogenesis, J. Steroid Biochem. Mol. Biol. 109 (2008) 323–330, https://doi.org/10.1016/J.JSBMB.2008.03.004.
[39] U.S. Environmental Protection Agency, Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Male Rats Assay, 2011. ⟨http://www.epa.gov/endo/⟩ (accessed March 10, 2022).
[40] U.S. Environmental Protection Agency, Validation of the Larval Amphibian Growth and Development Assay: Integrated Summary Report, 2013. ⟨https://docplayer.net/77411015-Validation-of-the-larval-amphibian-growth-and-development-assay-integrated-summary-report.html⟩ (accessed June 29, 2022).
[41] S.R. Hammes, Steroids and oocyte maturation - a new look at an old story, Mol. Endocrinol. 18 (2004) 769–775, https://doi.org/10.1210/me.2003-0317.
[42] A. Rajakumar, R. Singh, S. Chakrabarty, R. Muruganantkumar, C. Laldinsangi, Y. Prathibha, C.C. Sudhakumari, A. Dutta-Gupta, B. Senthilkumaran, Endosulfan and flutamide impair testicular development in the juvenile Asian catfish, Clarias batrachus, Aquat. Toxicol. 110-111 (2012) 123–132, https://doi.org/10.1016/J.AQUATOX.2011.12.018.
[43] H. Bhatia, A. Kumar, Does anti-androgen, flutamide cancel out the in vivo effects of the androgen, dihydrotestosterone on sexual development in juvenile Murray rainbowfish (Melanotaenia fluviatilis)? Aquat. Toxicol. 170 (2016) 72–80, https://doi.org/10.1016/J.AQUATOX.2015.11.010.