SHORT TERM SCIENTIFIC MISSION (STSM)
SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator

Action number: CA18221 - PEsticide RIsk AssessMent for Amphibians and Reptiles
STSM title: Suborganismal endpoints for reproductive toxicity: histological analysis of anuran gonads, nuptial pads and other organs
STSM start and end date: 24/02/2020 to 03/04/2020
Grantee name: Daniele Marini

PURPOSE OF THE STSM:

(max.200 words)
The purpose of our STSM was, firstly, to networking different professionals engaged with amphibians and reptiles. We propose to study endocrine disruptor pesticides able to unbalance reproductive traits and, consequently, fitness of amphibians. Our aim comprised also health of wild amphibians preliminarly studying in laboratory the possible predisposing role of pesticides as chronical environmental stressors associated with increased disease susceptibility and immunotoxicology. I was expected to learn more about anatomy and physiology of anurans, since I could carry out necropsies (based on availability of fresh material) and histology and, at the same time, enhance my skills on histology techniques and processing, my knowledge on larval and gonadal microscopic features and on endocrinology of amphibians. I was expected to improve my skills on anatomopathological interpretation of slides (and some other specific histological features). A literature review of the usefulness of histological endpoints (gonads, secondary sex characters) to assess reproductive toxicity in amphibians was proposed. Planned training was on sample preparation for histological analysis of the (primary and secondary) reproductive organs and on analysis of histological endpoints indicative of reproductive failure in *Xenopus tropicalis* frogs exposed to different endocrine disrupting substances including pesticides.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

(max.500 words)
Read about normal anathomy (including *Xenopus* 3D anatomy), histology and physiology of amphibians as well as about effects of exposure to endocrine disrupters chemicals (EDCs) on the reproductive system (primary and secondary reproductive organs including nuptial pads) in amphibians, precisely anurans. Start to write literature review on endpoints used in toxicity assays to measure effects of EDCs on reproductive function in amphibians. The main focus was on effects of anti-androgenic chemicals as they constitute the largest group of EDCs in general, and most likely also of endocrine active pesticides. More importance was given to histological endpoints in gonads and nuptial pads. The questions to adress were: Which endpoints have been used to measure effects of anti-androgenic chemicals? Which endpoints appear to be the most sensitive? Which one seems to be the most simple to use? Target pesticides were linuron, prochloraz, propiconazole and vinclozolin. Pharmaceutical flutamide was also addressed given that is used very often as anti-andreogenic positive control. Process of making histological slides and practice techniques: embedding in plastic/resin (glycol methacrylate Technovit 7100) for high-resolution histology (thin or semi-thin section of 2-3 µm), sectioning using a microtome with glass blade (MICROM), staining plastic sections (toluidine blue and Hematoxylin & Eosin stains), mounting histological slides, read them under optical microscope and microphotograph them with optical microscope (ZEISS), its digital camera (AxioCam Erc5s

– Zeiss) and dedicated software (AxioVisio Rel. 4.8). A little review on specific studied pesticide and on class of imidazole (conazole) short pharmacokinetical, pharmacodinamical and veterinary review as well as on nitrogen waste excretion of tadpoles and adult *Xenopus* anurans were carried out by grantee. In the middle of the mission was propose to the entire ecotoxicology group by the grantee a Journal Club with a topic on immunotoxicology, in order to start study this subject in search for innovation and new fronteers. Hence the topic was on spleen immotoxicological battery in cane toads (Fort *et al.*, 2016_ Splenic Immunotoxicity In Developing Cane Toads (*Rhinella marina*) From Bermuda. The journal touched Invasive Alien Species, the animal exposure, tissue preparation, morphological endpoints, immunological methods and endpoints applied on spleen, organs containing lymphoid/erithroid tissue in amphibians and question to address for further possible integrated investigations on pesticides.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

A review named as the STSM title ”Suborganismal endpoints for reproductive toxicity: histological analysis of anuran gonads, nuptial pads and other organs” was carried out by grantee. This review has as main topic histological endpoints used to measure effects of anti-androgenic compound/pesticide but it touches also: Normal Anatomy, Histology and Physiology; Effects of EDCs on reproductive system/ function in amphibians; Histological endpoints used to measure effects of EDCs. It has a discussion and conclusion based on: Histological endpoints used to measure effects of anti-androgenic chemicals; Sensitivity and Simplicity of histological endpoints for antiandrogenic compounds. This review it is attached to this STSM report as Annex I.

The training on histology permits to grantee to produce several complete and stained sections from samples of road killed toad (*Bufotes balearicus*) from his home institution. Some photomicrographs are inserted in the above –mentioned review.

The two little review were shared between the ”frog group”, as well as the master thesis of grantee, that helped for further brainstorming and insights on amphibian/reptile toxicology.

Furthermore during and after the Journal Club the group continued to brainstorm and included some pilot sampling of spleen to try integrate immunotoxicology in ongoing pesticide research.

FUTURE COLLABORATIONS (if applicable)

It is ongoing another call for a second STSM between grantee and Department of Environmental Toxicology at Uppsala University.

Supervisor and grantee think that immunotoxicity (including spleen and other organs) in amphibians is very relevant and they would be interested in going into this line of research. They are thinking to apply to research funding for such a project.

Suborganismal endpoints for reproductive toxicity:
histological analysis of anuran gonads, nuptial pads and other organs

Annex I of STSM Report

Daniele Marini, DVM

Short Term Scientific Missionary - PERIAMAR Cost Action

(CA18221 - PEsticide RIsk AssessMent for Amphibians and Reptiles)

Host Institution: Department of Environmental Toxicology, Uppsala University

Supervisor: Cecilia Berg, PhD, Associate Professor

9th April 2020

Contents

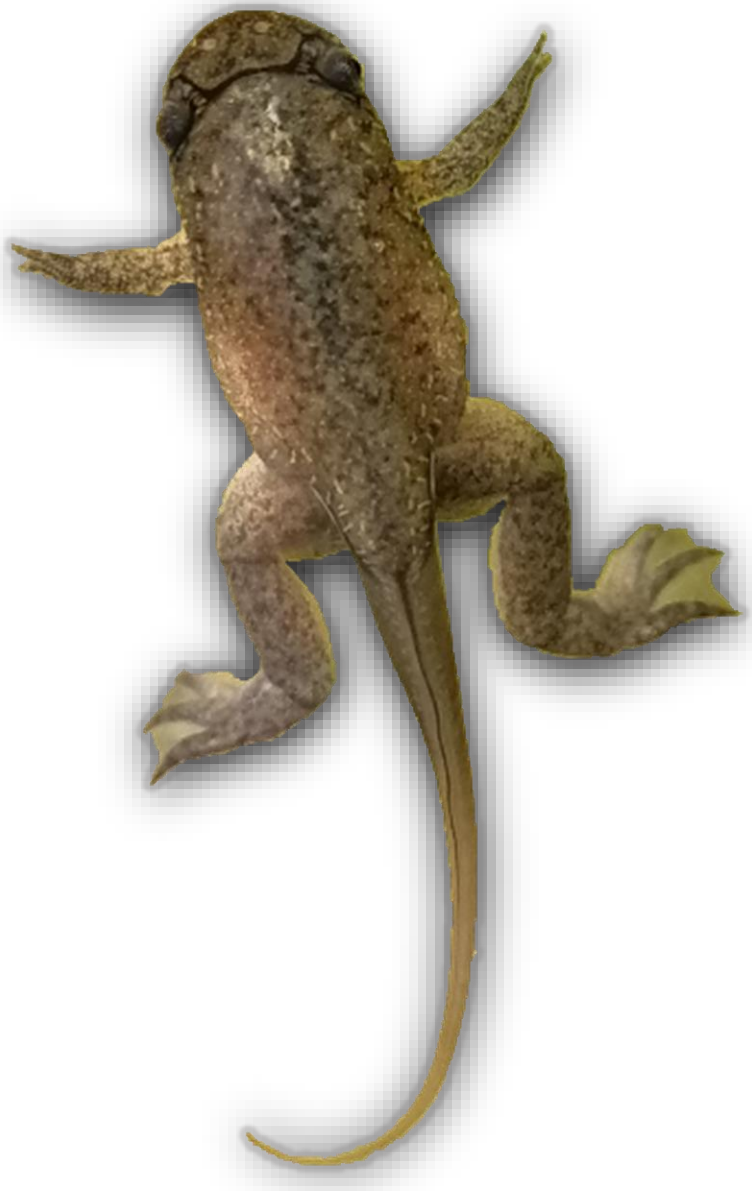

Figure 1: NF Stage 62 of *Xenopus tropicalis* tadpole. Photo credit Daniele Marini

1. Introduction

The total number of amphibian species is more than 8000 (AmphibiaWeb, 2020) and at least a third of this taxa is threatened with extinction or it is facing a serious population drop (Stuart *et al.* 2008; Bishop *et al.* 2012). Summarily, death and decreased recruitment are two sets of causes that can be used to explain this decline. Although sources of death as pathogens might be more straightforward, many factors may also affect directly or indirectly recruitment in a population (e.g. developmental or physiological factors) deviously resulting in reduced fitness and reproductive failure (Hayes *et al.*, 2010b). Precisely for this reason that it is worth to investigate the sub-lethal role of environmental pollutants on amphibian reproductive system. It is generally agreed that many xenobiotics used in agriculture, i.e. pesticides (herbicides and fungicides), may adversely affect the (vital) reproductive apparatus (Naz, 2005; Orton et al, 2018). One of most useful and important methods in toxicology, as well as one of the most complete and "self-sustaining" method that would lead us across several differential diagnosis and towards the most plausible causative agent, is the histology.

2. Normal Anatomy, Histology and Physiology

The major part of anurans is oviparous – others brood eggs in vocal sacs, stomachs, dorsal skin pouches, etc. and could be defined ovoviviparous or viviparous according to different authors – and practice external fertilization. Most of amphibians have genotypic sex determination and in the majority of anurans the female is the homogametic sex (XX) while the male is heterogametic (XY), although the sex chromosomes are not morphologically distinct; typically, the homogametic (default) sex does not require gonadal steroids for early differentiation (Norris & Carr, 2013).

Amphibian gonads are bipotential and in the early larval development undergo to sexual determination and differentiation (Norris & Carr, 2013). For example, *Xenopus tropicalis* is subjected to sex determination between the Nieuwkoop-Faber larval stage (NF – Nieuwkoop & Faber, 1956) 51 and 53 (El Jamil *et al.*, 2008) and to sex differentiation within NF 55-58 (Villalpando, & Merchant-Larios, 1990).

2.1 Gonads and germ cells

2.1.1 Ovary and oocytes

Anuran ovaries are hollow and sac-like organs (Norris & Carr, 2013) or lobular (Matsumoto & Ishii, 1992). At the peak of their growth the ovaries occupy the major part of the coelomic cavity, distending the body wall (Matsumoto & Ishii, 1992). They differ a lot between species and life stages (Fig. 2).

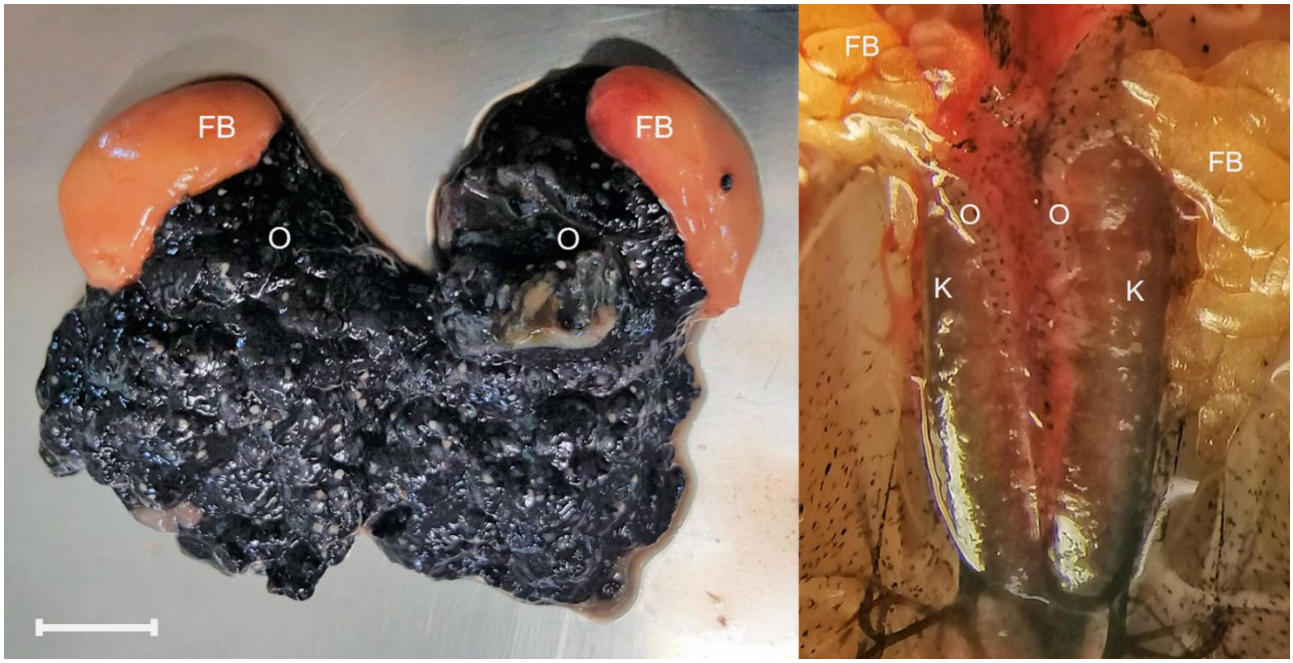

Figure 2: Difference between female gonads of different species at different ages. On the left: ovaries (ex situ) of an adult mature female of *Bufotes balearicus* (deceased close to the brumation period (fall), when ovarian follicular development is usually completed until suitable mating condition in the following late winter/early spring – Sept 2019, Abruzzi, ITA) during necropsy. Note the sac-like structure of ovaries. O: ovary; FB: fat body; Bar: 1 cm. On the right: ventral view of gonad mesonephros complexes (GMCs – in situ) of a neo-metamorphosed X. tropicalis (NF 66) during necropsy. Heart, lungs, liver, gastrointestinal tract and spleen have been removed to show better GMCs and annexed fat bodies. O: gonad phenotypically resembling ovary (rope-like structure); K: kidney; FB: fat body. Photo credit Daniele Marini.

Ovaries are covered by germinal epithelium, where the oogonia give rise to nests of oocyte. A single layer of granulosa cells composes the follicular epithelium deriving from the germinal one. Around the follicle is present a sequence of thecal cells, difficult to distinguish from other monolayer structures with light microscopy (Norris & Carr, 2013) (Fig. 3).

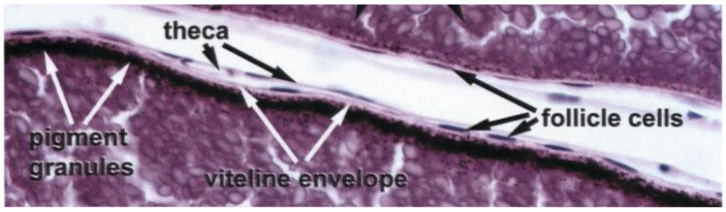

Figure 3: Oocyte cytoplasm and membrane of *X. laevis*. A thin thecal layer overlies flattened follicular cells on the extracellular side of the oocyte membrane. Modified from Wiechmann & Wirsig-Wiechmann (2003).

Different histological features of X. leavis oocyte development were adapted from Dumont (1972) and summarized by Cevasco and colleagues (2008): hereinafter some characteristics are introduced and simplified.

At the beginning of **pre-vitellogenic** stage (sub-stage I – oogonium) oocytes could be very small (50-300 µm) and symmetrical in shape having a thin follicular envelope; nucleus-germinal vescicle (GV) is central and cytoplasm homogeneously granular. At the sub-stage II (early) oocytes increasing dimentions (300-450 µm), the GV shape is more irregular, more nucleoli surround the nucleus and small granules are noticeable in peripheral cytoplasm. At the last sub-stage (III) oocytes are 450-600 µm and follicular cells are taller; cortical granules – and beneath them developing melanosomes – starting to appear at the periphery, as first yolk granules in form of spherical bodies; lampbrush chromosomes (LC) are present. Vitellogenesis in the liver and vitellogenin deposit in oocytes are sufficiently stimulated by estrogens (Norris & Carr, 2013), bringing oocytes to the second stage: the **vitellogenic** one. In the following sub-stage (IV – mid) the oocytes is 600-1000 µm and GV start moving toward animal pole with LC in its centre and nucleoli at its periphery. Successively (sub-stage V – late) the thickness of vitelline envelop is at its maximum and the follicular cells decrease in dimension. At the final **post-vitellogenic** stage (sub-stage VI or **diplotenic oocyte** according to Falconi *et al.*, 2001 and Säfholm *et al.*, 2012) oocyte reach 1200-1300 µm, follicles cells are flat with more concealed nuclei, cortical granules are aligned beneath external oolemma (*zona pellucida*) and GV is at the animal pole.

A classification of a post climax bufonid ovary is provided in Fig. 4, according to Orton, 2008.

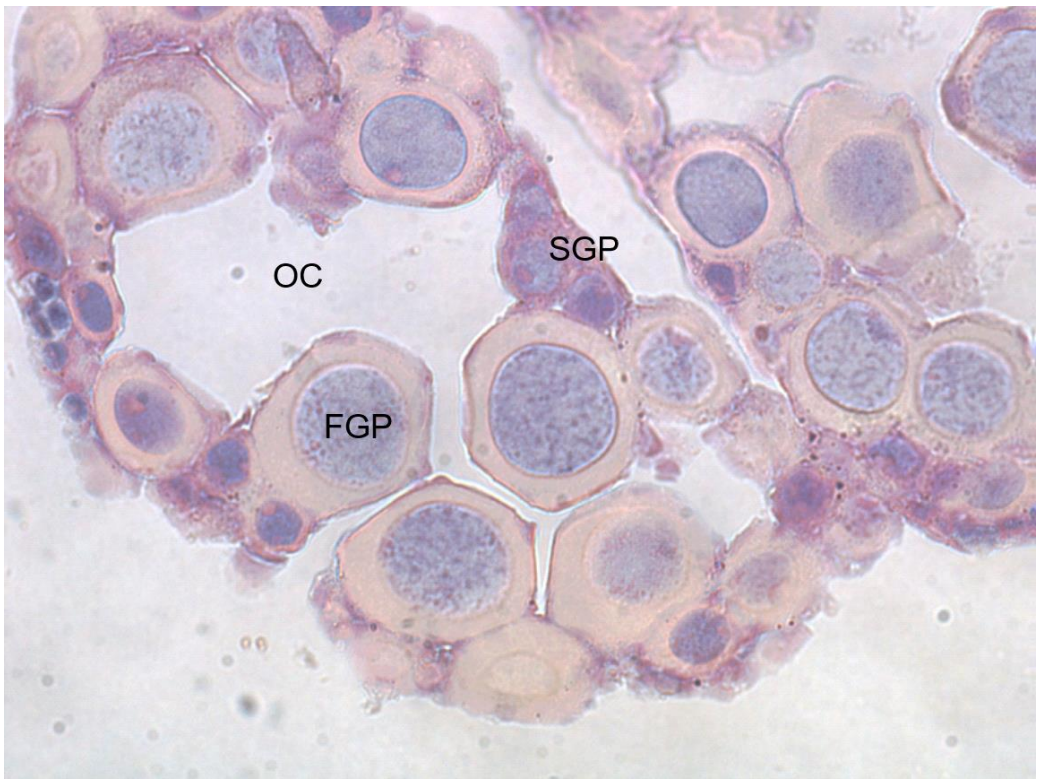

Figure 4: Ovaries from older metamorphs of *Bufo bufo* (Bufonidae). Classified according to Orton, 2008. Magnification 400x. OC: ovarian cavity; FGP: first growth phase oocyte; SGP: second growth phase oocyte. Modified from Orton, 2008.

Atresia is the controlled apoptotic process to which some oocytes are subjected becoming atretic.

2.1.2 Testes and male germ cells

Testes are ovoid to tubular organs. They usually lay down alongside the cranial part of the ipsilateral kidney (Matsumoto & Ishii, 1992) (Fig. 5).

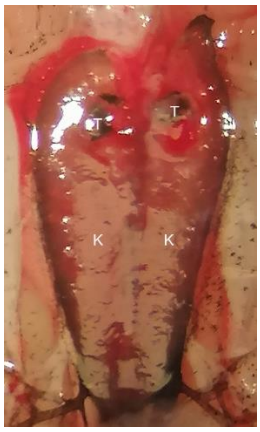

Figure 5: Ventral view of gonad mesonephros complexes (GMCs – in situ) of a neo-metamorphosed *X. tropicalis* (NF 66) during necropsy. Heart, lungs, liver, gastrointestinal tract, spleen and fat bodies have been removed to show better GMCs. T: gonad phenotypically resembling testis (ovoidal/lobular structure); K: kidney. Photo credit Daniele Marini.

Histologically the testis has a dense connective capsule (tunica albuginea) and a mediastinum receiving the mature gametes drained from seminiferous tubules (Wiechmann & Wirsig-Wiechmann, 2003). The seminiferous tubule is the functional unit of testis. Some authors consider anuran testis consisting of seminiferous lobules with a cystic pattern as other anamniotes (e.g. Norris & Carr, 2013). In any case, outside tubules (or lobules) is present an area consisting of loose connective tissue called intertubular space (Wiechmann & Wirsig-Wiechmann, 2003). Between this space and tubules and in the periphery of testis are situated Leydig cells, able to accumulate lipids, secrete androgens and conduct endocrinal activity (Norris & Carr, 2013). They have irregularly shaped nuclei and vacuolated cytoplasm containing lipid droplets (Wiechmann & Wirsig-Wiechmann, 2003). Within the tubules Sertoli cells are associated with immature germ cells and, in anamniotes, degenerate after spermiation, detaching and passing into the lumen; these cells may be in some cases source of androgens (Norris & Carr, 2013; Wiechmann & Wirsig-Wiechmann, 2003), they have a support function, also protecting germ cells (considered non-self) from autoimmunitary responses via tight junctions. Sertoli cells are large and eosinophilic, they enveloped spermatogonia in their basal portion (close to basal lamina) and they late spermatids attached to their apical (luminal) surface (Norris & Carr, 2013; Wiechmann & Wirsig-Wiechmann, 2003).

Different histological features of *X. leavis* male germ cells development (Fig. 6) were adapted from Kalt (1976) and summarized by Cevasco and colleagues (2008): hereinafter the stages are introduced and simplified, and they are described from immature toward mature gametes one as well as from the basal position toward luminal one.

The primary spermatogonia are large cells (sometimes bigger than 20 μm) with extensive nucleus and noticeable nucleoli; they are encircled by somatic cells. The following secondary spermatogonia are arranged in nest as cluster of cells with a round nucleus. Next in line, the leptotene primary spermatocyte is characterized by a large round nucleus with thin chromatin and single-double nucleolus. Zygotene primary spermatocytes have intermediate features with mildly condensed chromatids and region of nucleus. The pachytene primary spermatocyte has a thick chromatids and a single nucleolus while the following

diplotene primary spermatocyte is a very short stage. The secondary spermatocytes have a smaller nucleus with condensed chromatin while the next stage, the spermatid, has a dense nucleus (round to elliptical) and a developing acrosoma vesicle. The latest and mature germ cell, the spermatozoon, has distinctive elongated shape and is situated close to the lumen.

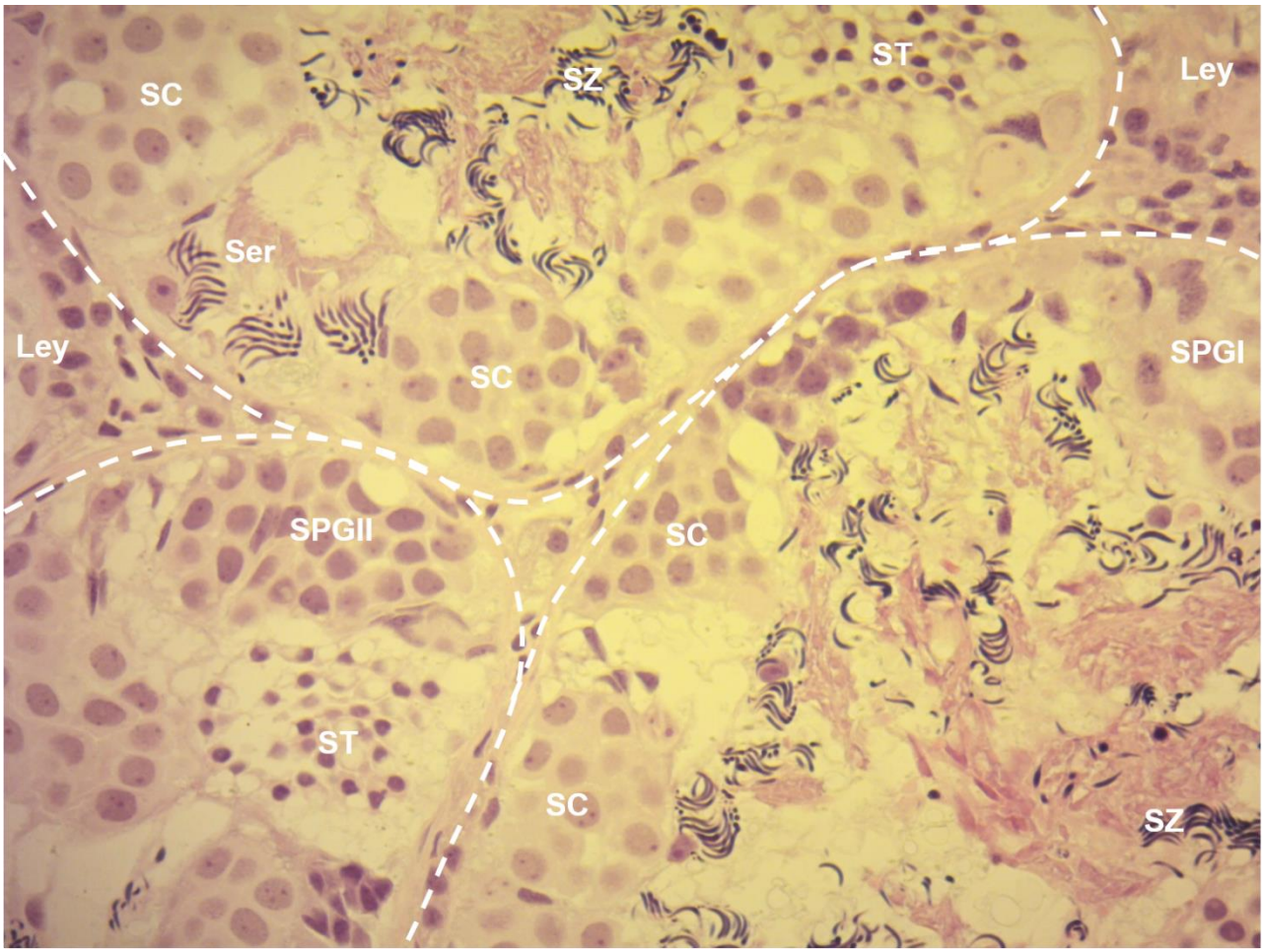

Figura 6: Photomicrograph of testis from adult *Xenopus tropicalis*. The image shows three seminiferous tubules (highlighted by dashed lines), different stages of male germ cells (within tubules) and adjacent interstitial tissue/space. SPGI: primary spermatogonia; SPGII: secondary spermatogonia; SC: spermatocytes; ST: spermatids; SZ: spermatozoa. Ley: interstitial connective tissue/space where Leydig cells are found; Ser: intertubular (basal)space where eosinophilic Sertoli cell are found. Hematoxylin & Eosin stain. Magnification 400x. Photo credit Daniele Marini & Uppsala University.

A recent study described prespermatogenesis (male germ cell development during larval stage) and early stages of spermatogenesis of two *Pelophylax* sp. (Ranidae), further discriminating two classes of primary spermatogonia and bringing to light additional classification of spermatogenic cells in these taxa (Haczkiewicz *et al.*, 2017).

Usually during normal development, all germ cells associated with a single spermatocyst – maintaining a circular to ovoid shape – mature at a similar rate (Haselman *et al.*, 2018).

Male mature gametes are delivered through efferent ductules and the kidney to the Wolffian duct (Matsumoto & Ishii, 1992).

2.2 Reproductive ducts

In amphibians are noticeable two kinds of reproductive ducts during the early life stages (e.g. Jansson et al 2015). Müllerian duct is retained in female and develops into a functional oviduct and – in some species – ovisac/uterus, while in the male, depending on the species, it can remain rudimental or disappear. Wolffian duct develops the function of spermatic and urinary duct in male amphibians, while in females is always retained as urinary duct draining the mesonephric kidney (Norris & Carr, 2013).

The oviduct in anuran females is a large convolutes tubular organ and, in some species, the caudal segment is expanded into ovisac (or uterus) where eggs may be retained till spawning (Matsumoto & Ishii, 1992). Histologically it is surrounded by a layer of connective tissue and a fibrous stroma supports basally the glands (Fig. 7); in some specialized oviductal area these tapered glands secrete into the lumen the jelly coat that will accompany the oocytes toward – ovisac and – cloaca (Matsumoto & Ishii, 1992; Wiechmann & Wirsig-Wiechmann, 2003).

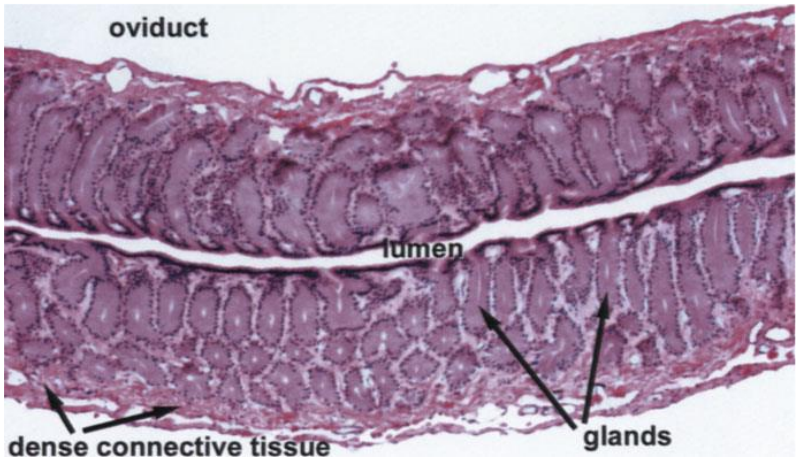

Figure 7: Oviduct of *Xenoupus laevis*: longitudinal section. Lumen, connective tissue and secretive glands are visible. Wiechmann & Wirsig-Wiechmann (2003).

2.3 Other organs in the reproductive system

2.3.1 Fat bodies

Fat bodies (Fig. 2) are exclusive adipose amphibian structures of both sexes, situated adjacent to gonads. Lipidic compounds storage for oocyte growth and neo-synthesis of steroids for sex structures were hypothesized as some of their functions (Norris & Carr, 2013).

2.3.2 Bidder organ

Some male bufonid anuran (Bufonidae) has rudimentary ovaries or Bidder's organs. Histologically, it is formed by a compact mass of small oocytes, subjected to a limited seasonal growth and degeneration cycle correlated with the testicular cycle. These bidderian oocytes do not reach the vitellogenic stage (Norris & Carr, 2013).

2.4 Secondary sexual physical/phenotypic characteristics

Secondary sex characteristics (SSCs), or sex accessory structures, are not directly part of reproductive system as the primary ones. In amphibians they include nuptial pads and enlarged fore limbs in males and a protruding cloaca in females. These features appear generally during puberty or at sexual maturity and are particularly evident in the sexually dimorphic phenotypic traits (see below).

2.4.1 Nuptial pads

Male anuran amphibians have a modified epidermal and dermal tissues generally located on the first digits: this second sexual character is called nuptial pad. They are a commonly observable at naked eye as thickening of the skin with a rough surface and a different (darker) colour (Fig. 8).

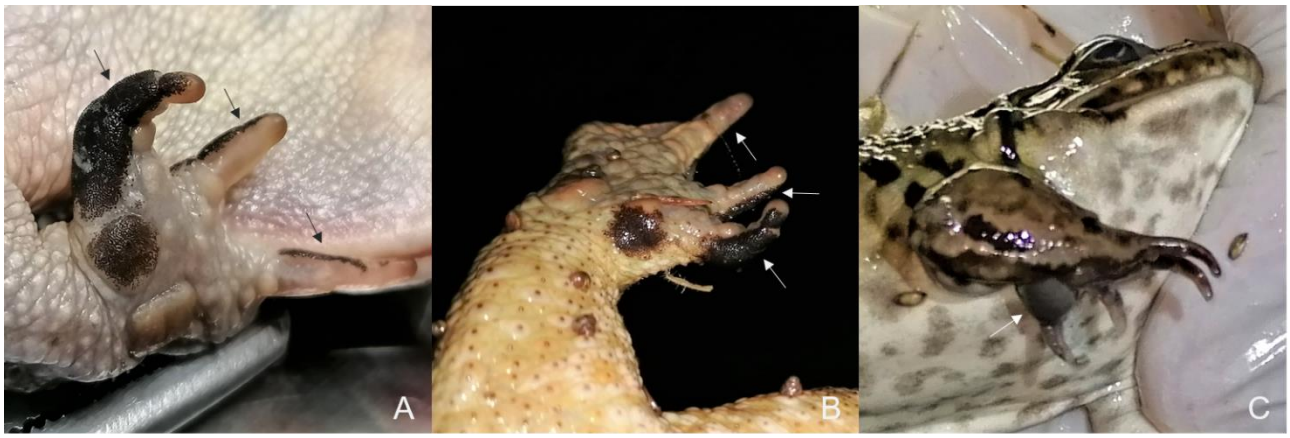

Figura 8: Macroscopic view of nuptial pads (NPs) from different species during mating season. A: *Bufotes balearicus* (Bufonidae – road killed). B: *Bufo bufo* (Bufonidae). C: *Pelophylax* sp. (Ranidae). Note the presence of NPs (arrows): in Bufonidae (A; B) NPs are noticeable in each medial surface of the first three digits, while in Ranidae (C) NP is present only in the medial surface of the first digit. The aspect of NPs is rough and dark. Photo credit Daniele Marini.

In nuptial pad (NP) derma there is thicker a strata spongiosum and compactum compared to adjacent skin; here we can find specialized mucous glands (breeding glands according to Orton *et al.*, 2018) having an intraepidermal duct (Luna *et al.* 2018). Extreme modification of the cellular layers of stratum spinosum (increased) and of strata spinosum and corneum morphology (turgid cuboidal cells and thicker keratinized cells, respectively) are seen in epidermis (Luna *et al.* 2018). Different morphologies let recognize three kinds of NPs (Luna *et al.* 2018): NPs with papillary epidermal projections (e.g. Fig. 9), NPs with non-papillary epidermal projections and smooth NPs. Xenopus tropicalis is part of the first group having spicule-shaped papillae, the smallest of the papillae evident only at higher magnification but of easier identification in Xenopus spp. due to dark-coloured stratum corneum (Luna *et al.* 2018).

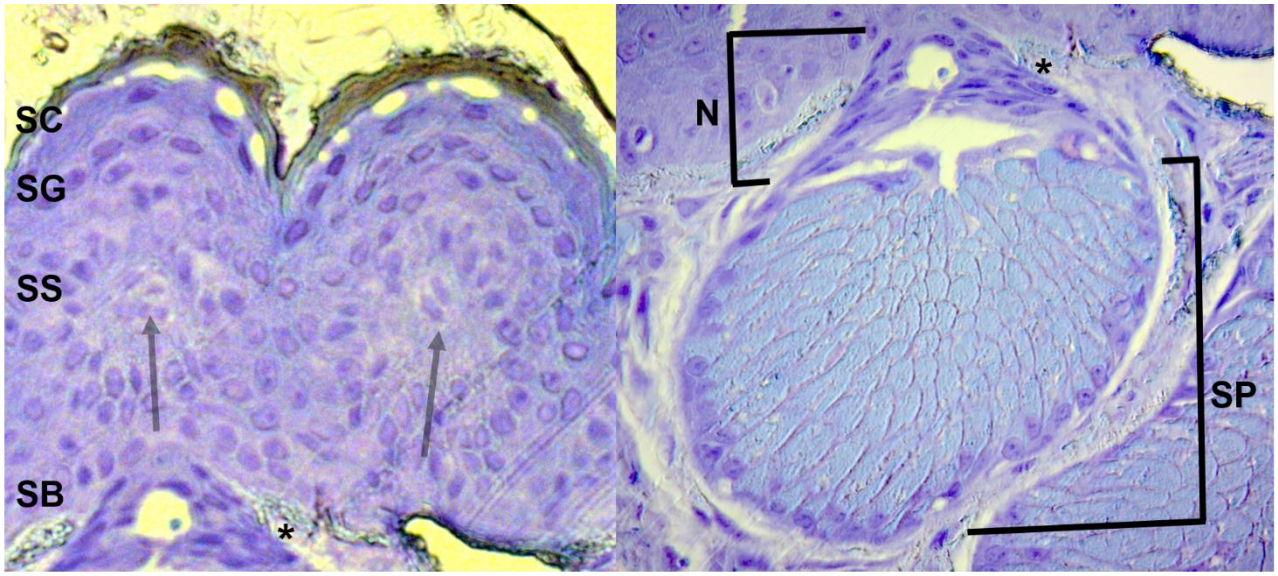

Figure 9: Photomicrographs of NP histological structures (according to Luna et al., 2018) of road killed male *Bufotes balearicus* (Bufonidae). This species has papillary epidermal projections with ornamentations in the apices of the papillae, in both the stratum granulosum and the stratum corneum, as shown on the left: evagination of epidermal components are highlighted by semi-transparent arrows and the apices (of neck portion) of specialized mucous gland is at the bottom [note that the micrographs are from the same slide and the asterisk (*) has been used as a landmark giving that right image is just ventral to the left]. On the right an active specialized mucous gland (breeding glands according to Orton et al., 2018): the neck has 2 to 4 layers of cells while secretory portion is formed by a monolayer of cells filling the lumen with their mucous secretion. Semi-thin section of resin embedded III digit. Toluidine blue stain. Magnification 400x. N: neck; SB: stratum basale; SC: stratum corneum; SG: stratum granulosum; SP: secretory portion; SS: stratum spinosum. Photo credit Daniele Marini & Uppsala University.

NPs help mechanically (more friction) the males holding the female during amplexus (commonly axillary in ranids and inguinal in X. leavis - Kyriakopoulou-Sklavounou *et al.*, 2012), mostly if aquatic. Relatively recently, proteins secreted during mating period by NPs of *Rana temporaria* named amplexins were discovered: they might someway influence frog courtship (Willaert *et al.*, 2013). Keratinized epidermis and nuptial glands show androgen receptors and respond to androgen in most species (Emerson at al., 1999), very likely only in adults (mostly in mating season - Norris & Carr, 2013) and in juveniles reaching the puberty.

2.4.2 Larynx

Into the buccal cavity of frogs, just below the oral portion of the esophagus, is noticeable the glottis that open directly within larynx and trachea. Anurans larynx possess a thyrohyal, a cricoid and a pair of arytenoid cartilages, lacking of a true epiglottis (glottis appear as a simple fissure on the sagittal plane). The major part of frogs vocalizes with vibrations of vocal cords subsequent to the opening of the glottis by dilator laryngis muscle, instead in Pipidae (*Xenopus* sp.) vocal cords are absent and the sound is produced by arytenoids (Boyds *et al.*, 1999). In latter taxa vocal apparatus is substantially different with dilator muscle and cartilages enlarged and providing an important role (Sassoon & Kelley, 1986; Boyds *et al.*, 1999). In several number of species both anuran sexes vocalize, but usually only males emit calls for different strategies. These additional functions appear as a sexual dimorphism of the vocal apparatus, particularly in the size, number of fibres and metabolic properties of dilator laryngis muscle (e.g. Sassoon & Kelley,

1986; Boyds *et al.*, 1999). It was revealed that this kind of muscle possess androgen receptors (at higher level and over a longer ontogenetic period in males in X. laevis) and that in post metamorphose development (likely the first 6 months in X. laevis) or during adulthood (depending on species) androgens may control anatomic and enzymatic sexual dimorphisms of larynx (Sassoon & Kelley, 1986; Kelley *et al.*, 1989; Boyds *et al.*, 1999).

2.4.3 External oblique muscle

Anurans, as many other vertebrates, have an internal and an external oblique muscles located in the abdominal (or coelomic) wall (Fig. 10). In some anuran families (e.g. in male Hylidae during breeding season), more than in others (e.g. Pipidae), they are implied in vocalization since their contraction is followed by the passage of air and the vibration of assigned structures in the larynx (see above; Emerson *et al.*, 1999). It was revealed that the external oblique muscle (mostly in males but also in females) of the hylid *Pseudacris triseriata* had significantly high number of androgen receptor compared to muscles not linked with reproduction (i.e. gastrocnemius muscles – Emerson *et al.*, 1999).

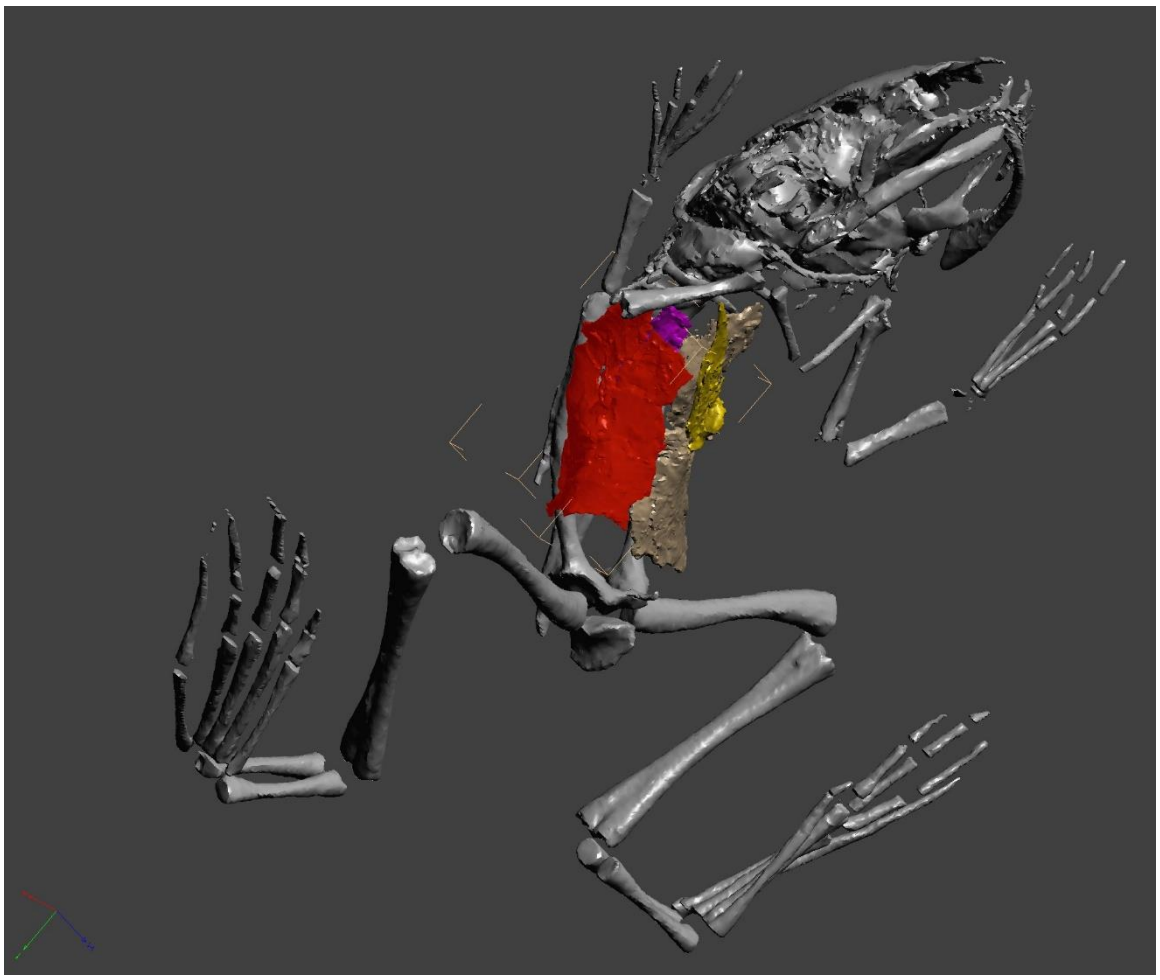

Figure 10: Ventral-right lateral view of 3D anatomy model of *X. laevis*. The right external oblique muscle is highlighted in bright red into a box defining borders. It is noticeable the right rectus abdominis superficialis (yellow), the right rectus abdominis profundus (bright brown) and the right transversus abdominis (purple). Image from interactive 3D PDF of the digitally segmented musculature of Xenopus laevis (S2 from Porro et al., 2017).

3. Effects of EDCs on reproductive system/function in amphibians

An endocrine disrupting chemical (EDC) is defined as an exogenous substance that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny (WHO/IPCS 2002). EDCs influence endocrine physiology at different levels, including mimicking by binding to receptors, blocking receptor binding, disrupting hematic binding proteins or catalysis/pathway of hormones/enzymes (Naz, 2005).

The impairment of sexual function/fertility in adults and/or developmental toxicity in progeny is defined as reproductive toxicity (EFSA, 2018). Hence, to test this toxicity in advanced or early life stages it is necessary to address investigation to impaired fertility and reproductive organ changes in adulthood, or to viability, sex ratio and growth in larval stages (EFSA, 2018).

Generally in anamniotes the sex determination and sex differentiation occur during the early larval development. The precise time intervals in the course of the larva undergo to sexual determination and differentiation (see Chapter 2) are windows of sensitivity, and their width depend on the species (Norris & Carr, 2013). Nonetheless, these time spans could be of major concern when the developing animals are exposed to EDCs, thus becoming critical periods. This fact is even worst in anamniotes (as amphibians) since "their amnios" could be considered the (likely polluted) environmental water!

Moreover, since the development of gametes and gonads (sex differentiation) occurs during larval stages in amphibians (EFSA, 2018), the EDCs exposure during prejuvenile stages may further affect the fitness of future adult individuals and, consequently, population dynamics. Beyond, it is supposed that some chemical treatment may also affect the sex defining pattern of mRNA expression, therefore distinguish genetic sex (Orton *et al.*, 2018) and disrupting the sex determination. This organizational effect derived by exogenous compounds often results in permanent designation of sex and sexual characteristics in form of complete or partial (intersexes) sex reversal leading to skewed sex ratios (Hayes et al 2002; Pettersson and Berg 2007; Gyllenhammar *et al.*, 2009; Orton et al 2018). For this reason, EDCs probably are the major agents causing the increase of reproductive, developmental and pathological dysfunctions.

3.1 Effects of anti-androgenic chemicals

Endogenous androgens (from Greek andr-, meaning "man") are steroidal hormones that, by binding to androgen receptors (ARs), control the development/maintenance of male gonads and SSCs in vertebrates; typically, they are synthesized in the testes, the ovaries, and the adrenal glands (Naz, 2005; Norris & Carr, 2013). Although scientific community has mostly begun to devote itself on EDCs' inhibitory actions of the AR in the lasts years of 90's (van Wyk *et al.*, 2003), nowadays anti-androgenic compounds are considered the most prevalent type of EDCs (Orton *et al.*, 2018).The nonsteroidal pharmaceutical flutamide could be considered the "pioneer" of anti-androgens, investigated by Gladue and Clemens in 1980, discovered to be a competitive inhibitor of AR, and thereafter used as a positive control for anti-androgenic activity of chemicals in toxicological studies (e.g. Chapter 4.1.1; van Wyk *et al.*, 2003; Orton *et al.*, 2018).

Exposure to anti-androgenic chemicals usually leads to demasculinization of males – abnormal (weak to absent) male sex differentiation, puberty achievement or SSCs display, contrary of virilisation – by blocking AR binding. However, it cannot be assumed that all anti-androgenic EDCs the have the exactly same mechanism of action (MOA – van Wyk *et al.*, 2003; Orton *et al.*, 2018).

Hereinafter (Chapter 4.1) are reviewed specific chemicals/pesticides with mentions about chemical composition and MOAs.

4. Histological endpoints used to measure effects of EDCs

Developmental, reproductive and developmental reproductive pathologies in amphibians can be assessed via histology (eg. Säfholm et la 2012; Gyllenhammar 2009; Kvarnryd et al 2011; Säfholm et al 2016; Orton *et al.*, 2018). Regarding reproductive disorders/disruptions, the histopathological investigation of primary sexual characteristic is the earliest permitted in metamorphs (NF 66), while for secondary sexual characteristics typically in adulthood (See Table 1; eg. Säfholm et al 2012; Orton *et al.*, 2018).

An endpoint for ECDs commonly used in amphibian laboratory studies is the histological investigation complete or partial sex reversal and the consequent alteration of the sex ratio relative to the control group (Pettersson & Berg, 2007; EFSA, 2018) while a frequently used histological endpoint in wild amphibian studies is the presence of male intersex or ovotestis albeit it seems to be highly biased by different gonadal differentiation patterns among amphibian species (EFSA, 2018). In some populations of species such as *Rana temporaria* an intersexual stage occurs naturally, see e.g. Pettersson and Berg (2007) and the references therein.

Life stage (sampled)	Endpoint measured	Endpoint for	Exposure period	Age/larval stage sampled	Reference
Juvenile	Phenotypic sex ratio (gonadal histology)	ED mode of action; targeting oestrogen-, androgen signalling pathways	Larvae	At completed metamorphosis, NF66 in *Xenopus* sp., 2 months postmetamorphosis in *X. laevis*	*X. laevis*: Kloas *et al.* (1999), Haselman *et al.* (2018) *X. tropicalis*: Pettersson and Berg (2007)
	Histopathology of Müllerian and Wolffian ducts	Potential reproductive toxicity	Larvae or juveniles	1 month postmetamorphosis in *X. tropicalis*, 2 months postmetamorphosis in *X. laevis*	Jansson *et al.* (2016), Säfholm *et al.* (2016), Haselman *et al.* (2018)
	Gonadal maturity: histomorphometry proportions of germ cell stages (oogenesis and spermatogenesis)	Potential reproductive toxicity (gonadal development and maturation)	Larvae or juveniles	1 month postmetamorphosis in *X. tropicalis*, 2 months postmetamorphosis in *X. laevis*	Säfholm *et al.* (2016) Haselman *et al.* (2018)
Adult	Larynx histopathology	ED mode of action; targeting androgen signalling	Larvae	1–2 years in *X. laevis*, 4–6 months in *X. tropicalis*	Sassoon & Kelley (1986), Tobias *et al.* (1993), Hayes *et al.* (2010a)
	Male histopathology: testis including spermatogenesis	Reproductive toxicity	Larvae or adults	1–2 years in *X. laevis*, 4–6–8 months in *X. tropicalis*	Knechtges *et al.* (2007) Cevasco *et al.* (2008), Gyllenhammar *et al.* (2009), Hayes *et al.* (2010a), Berger *et al.* (2011), Kvamryd *et al.* (2011), Orton *et al.* (2018)
	Female histopathology: ovary including oogenesis, oviduct	Reproductive toxicity	Larvae or adults	1–2 years in *X. laevis*, 6–8 months in *X. tropicalis*	Knechtges *et al.* (2007) Cevasco *et al.* (2008), Säfholm *et al.* (2012, 2014, 2016)
	Expression of secondary sex characters (nuptial pads, cloacal size)	ED mode of action; targeting sex hormone signalling pathways	Larvae or adults	1–2 years in *X. laevis*, 4–6 months in *X. tropicalis*	Wyk *et al.* (2003), Säfholm *et al.* (2012), Orton *et al.* (2018)

Table 1: Amphibian test histological endpoints for reproductive and developmental reproductive toxicity at juvenile and adult life stages. Larval stages are referred to Nieuwkoop – Faber (NF) staging system (Nieuwkoop and Faber, 1956). Modified and implemented from EFSA 2018

4.1 Histological endpoints used to measure effects of specific anti-androgenic compound/pesticide

4.1.1 Flutamide

Flutamide is a nonsteroidal anti-androgen, well characterized last century (Gladue & Clemens, 1980). Its MOA concern AR binding and inhibition of androgen-regulated genes transcription subsequent to passage into the nucleus (Kelce *et al.*, 1997).

Cevasco and colleagues (2008) exposed adult X. leavis to several chemicals, including flutamide (2.8 µg/L), in 2 case groups of 8 animals (sex ratio 1:1) for 4 weeks. Results showed that seminiferous tubule diameters were reduced in testes of all the frogs exposed to flutamide. Histomorphological alterations were not find in testes of flutamide exposed males, except in one individual that presented thickening of interlobular walls, increasing number of Sertoli cells, cell nests detaching from the wall and lumen filled with scattered cellular debris and blood cells (Fig. 11). The 50% and 16,7% of exposed individuals showed increased and decreased average number of cell nests per tubule cross section, respectively, compared to that of controls. The pharmaceutical compound did not induced ovotestes in males (Cevasco *et al.* 2008).

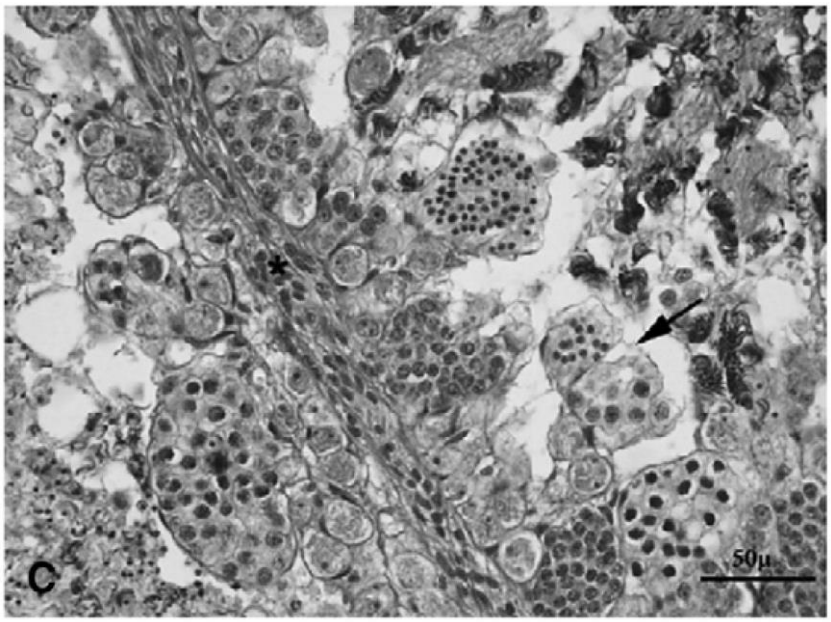

Figure 11: Magnification of the interlobular wall (*) between two seminiferous tubules from one FLU exposed male testis. Increasing number of Sertoli cells and numerous cell nests detaching from the wall (arrow) are evident. (Cevasco et al 2008)

Knechtges and collegues (2007) performed a flow-through exposure of *X. tropicalis* from NF 46 to 30 weeks to 10 mg/L o flutamide. At gross necropsy the define the condition of ''non recognizable gonadal tissue'' in exposed individuals that did not show testes, assuming that they were genetic males. They did not observe germ cell degeneration in both control and exposed, but they occasionally noticed ovotestes and mutated germ cells within seminiferous tubules, interstitial cellular infiltrations and interstitial fibrosis in both groups. None other histological alterations were observed.

A compressed pellet of flutamide (0,5 mg/g BW) was implanted for 3 months into the dorsal sac of newly metamorphosed to assessed effect of anti-androgen on laryngeal muscle fibres addiction during growth. At the end of exposure, individuals were euthanized, larynges were dissected, fixed, resin embedded and transversely cut to permit counting of muscle fibres of dilator laryngis from each side. Control male fibres were around two times higher than control female ones, while flutamide-treated males values were less than those of control males and not significantly different from control and exposed females (Sassoon & Kelley, 1986).

4.1.2 Linuron

Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) is a phenylurea (urea-based) herbicide considered to have anti-androgenic effects (in vitro and in vivo) binding directly to the AR and competitive inhibiting gene activation of this receptor (Wilson *et al.*, 2008; Marlatt & Martyniuk, 2017).

Effects of this urea-based compound during a life cycle/reproductive *Xenopus tropicalis* test system were reported by Orton and colleagues (2018). They exposed fertilized eggs until metamorphosis to 9 or 45 µg/L of linuron and pharmaceutical flutamide, as positive control: tadpoles were sub-sampled analysing the genetic expression of gonadal sex during sensitive windows of sexual development (NF 55-58) and comparing it thorough histopathology of metamorphs (NF 66) gonad mesonephros complex. Remaining adults exposed during larval stage were reared in substance-free water until sexual maturity (6 months post-metamorphosis): some adult females (ad interim) were euthanized to carry out ovarian histomorphology, some adult females underwent breeding, and some adult males were sub-sampled directly after breeding for gonadal histomorphology. Moreover, nuptial pads histomorphology was conducted in all males reaching sexual maturity (Orton *et al.*, 2018). For histological endpoint methodology see Fig. 12, 13, 14 (and annexed captions) from Supplementary material of Orton *et al.*, 2018.
In all neo-metamorphosed females from high linuron group (50% in control females) were found diplotenic oocytes (post-vitellogenic stage or sub-stage VI according to Cevasco *et al.*, 2008) but no differences were found in testicles (Fig. 12). In adults: ovarian maturity was not affected by exposure while the number of spermatogonia per seminiferous tubule were doubled compared to control males (Fig. 13); diameter of tubule was larger compared to control in exposed to higher concentration of linuron; increased number of spermatogonia was revealed in flutamide and lower concentration of linuron exposed ad interim or not (post-breeding males).

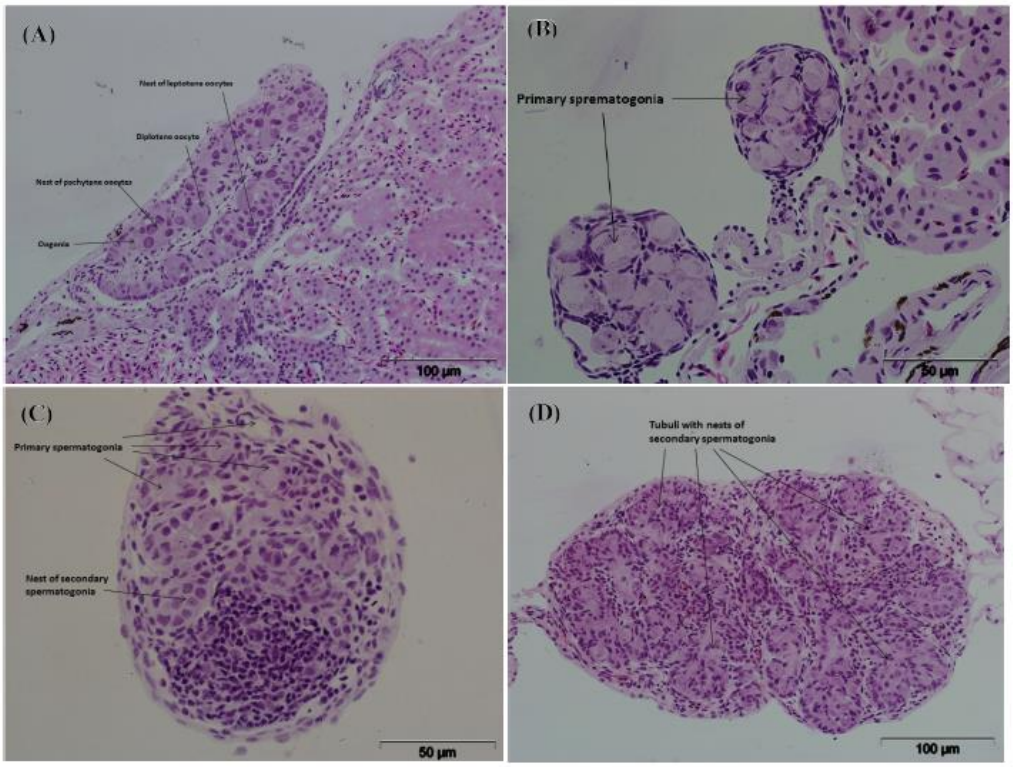

Figure 12: Photomicrograph of gonad slides from metamorphosed western clawed frog (NF 66). A: ovary containing different stage oocyte. B: Testis at Stage 1- primary spermatogonia without tubules. C: testes at stage 2- secondary spermatogonia without tubules. D: testes in testicular maturation at Stage 3- secondary spermatogonia with tubules. SPG: spermatogonia; SC: spermatocytes; ST: spermatids; SZ: spermatozoa. Hematoxylin & Eosin stain. From Orton et al., 2018.

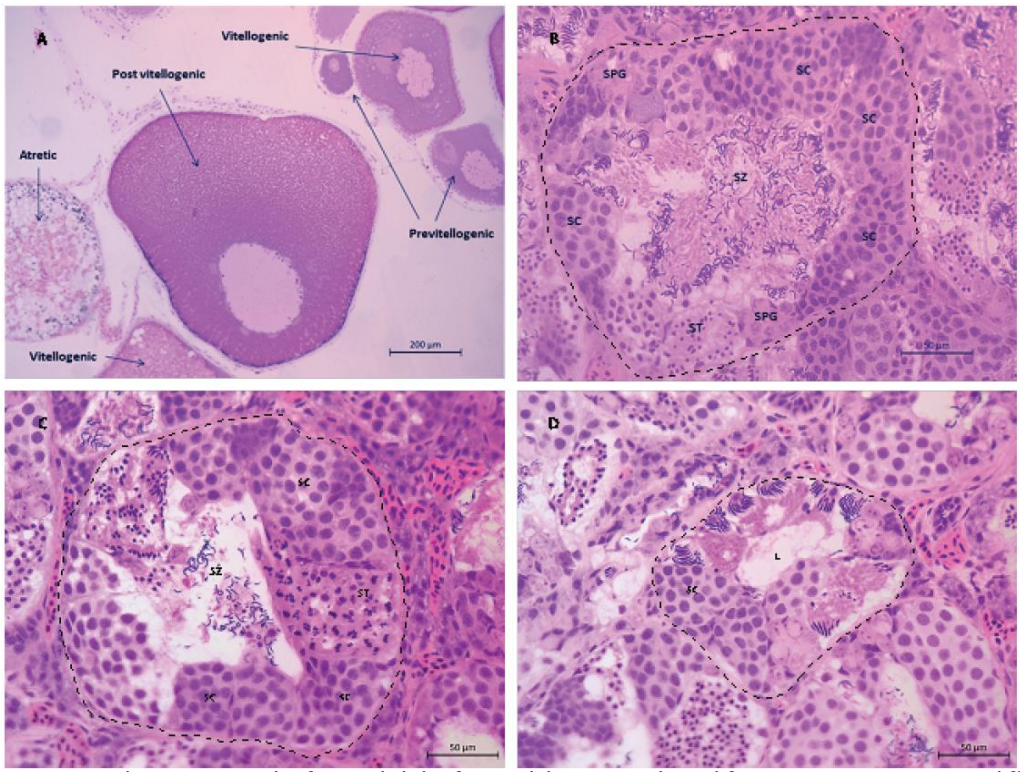

Figure 13: Photomicrograph of gonad slides from adult western clawed frog. A: ovary containing different stage oocyte. B: Score 3- spermatozoa full filled seminiferous tubule. C: Score 2- a seminiferous tubule half filled with spermatozoa. D: Score 1- a seminiferous tubule without luminal spermatozoa. SPG: spermatogonia; SC: spermatocytes; ST: spermatids; SZ: spermatozoa. L: lumen. Hematoxylin & Eosin stain. From Orton et al., 2018.

Additionally, also nuptial pads histological endpoints response not following a monotonic dose-response relationship: a decrease in size, number of breeding glands (specialized mucous glands according to Luna *et al.* 2018) and number of hooks (spicule-shaped papillae according to Luna *et al.*, 2018) was observed in linuron low exposure, while an increase in size was noticed in linuron high treatment and flutamide exposure (beyond a darker colour in high linuron and higher number of hooks in flutamide; Orton *et al.*, 2018 - Fig. 14).

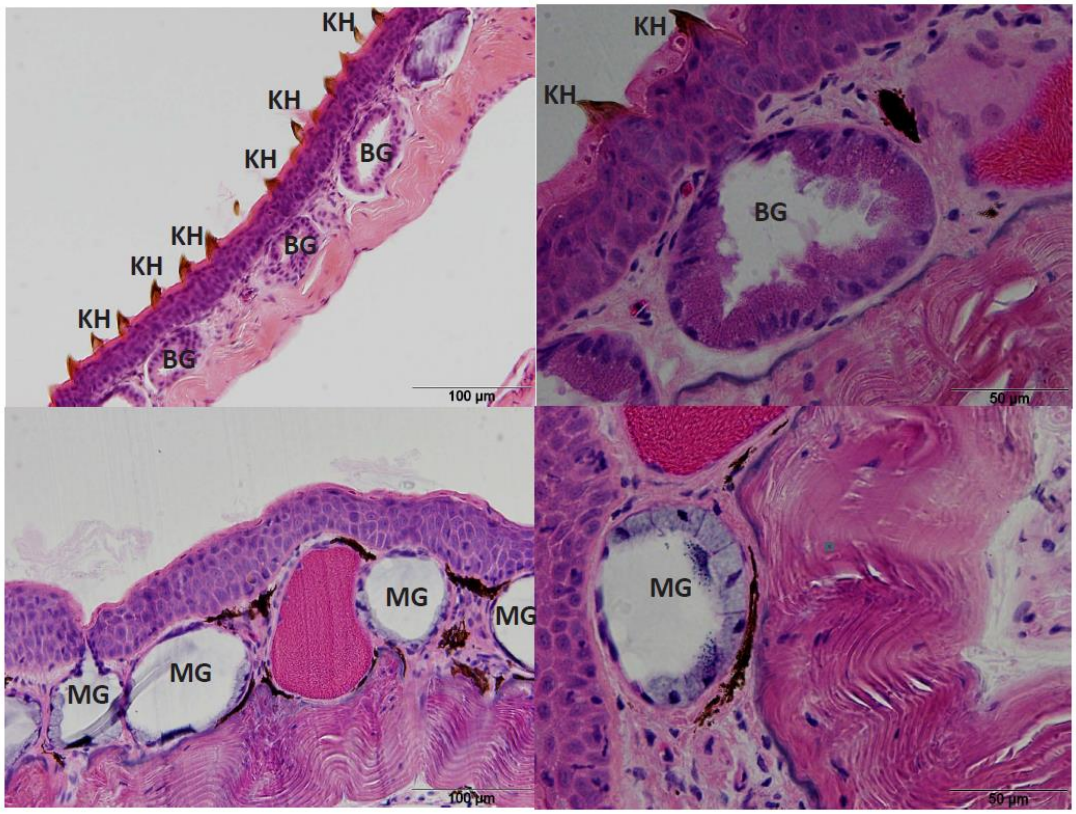

Figure 14: Photomicrographs of arm sections from adult western clawed frog. A: nuptial pad tegumentum where are visible many spicule-shaped papillae (KH) and specialized mucous glands(BG) (Magnification: 100x). B: Slide showing a BG and KH (Magnification: 400x). C: arm showing ordinary mucous grands (MG) (Magnification: 200x). D: Tissue revealing a MG (Magnification 400x). KH: keratinised hooks; BG: breeding (specialized mucous) gland; MG: (ordinary) mucous gland. Hematoxylin & Eosin stain. Orton et al., 2018.

4.1.3 Vincozolin

Vinclozolin and procymidone are AR antagonists dicarboximide fungicides. The MOA of vinclozolin, working together with its metabolites, is due to competitive inhibition of androgens to AR of different vertebrate species, and, similar to flutamide, to directly alter the expression of androgen-dependent genes (Kelce *et al.*, 1997; Gray *et al.*, 2005). Moreover, this compound with its final products synergetically act to disrupt other endocrinal/reproductive mechanisms (Molina-Molina *et al.*, 2006).

van Wyk *et al.* (2003) submitted adult wild captured *X. laevis* to repetitive intraperitoneal injection – q7d x 3 times – of 5 chemicals, including vincozolin (100 µg/g/week) and flutamide (100 µg/g/week – used as positive control), during 28 days of stalling before euthanasia. Tissue sections surrounding nuptial pads (3 X 3 mm) were excised and routinely fixed, embedded (plastic), sectioned and stained (H&E as counterstain) for histological and morphometrical examination. The mean of nuptial pads epithelium heights of male housed the entire period and treated with vincozolin and flutamide decrease approx. by half and two thirds, respectively, compared to negative control group. Males epidermis in these treatment groups presented for the most part absence of keratinized epidermal hooks and consistently lower breeding gland cell (male gland active or not) in number and volume. In both sexes gland epitelium cells were mostly cuboidal or squamous with mostly indistinct cell boundaries (staining weakly) and no visible (eosinophilic) cytoplasmatic granules instead of single layer columnar cells with well-defined cellular boundaries and apical cytoplasmatic granules (van Wyk *et al.*, 2003).

The findings of van Wyk and collegues (2003) confirmed the reproductive disruption of this dicarboximide fungicide in adult frogs and gave the preliminary indication on the use of nuptial pads/glands as sensitive biomarker of demasculinization (anti-androgenical activity) starting from the gland epithelium height and gland cross section correlated with gland activity/dysfunction.

4.1.4 Prochloraz

Prochloraz is an imidazole (conazole) fungicide which acts as an AR antagonist and as an inhibitor of steroidogenesis enzymes CYP19 aromatase and CYP 17α-hydroxylase/17,20-lyase (reviewed in Haselman *et al.*, 2018).

A slightly modified LAGDA study was used to evaluate effect on reproductive development of juvenile gonsds and reproductive ducts of X. laevis exposed – from less 1d post-fertilization to NF 66 (interim) or 2 monts postmetamorphosis – to 4 different concentrations of this imidazole compound (Haselman *et al.*, 2018). At the histological analysis – applied to late juveniles – in all exposed groups females showed ovarian hypoplasia, mononuclear cellular infiltrate and granulomatous inflammation. Alterations of histology were seen in male gonads mainly at the highest concentration of xenobiotic (180 µg/L): germinal epithelial thinning/loss with or without apoptosis or necrosis (higher prevalence in higher treatment); dose-dependent asynchronous development of germ cells within a spermatocyst (with some spermatocysts irregular in shape); interstitial cell hypertrophy and hyperplasia, interstitial fibrosis and mononuclear cellular infiltrate (higher incidence in high dose exposure). An inhibition of male Müllerian duct regression was noted in concentration-dependent manner, as well as exceptional partially maturation, while female showed accelerated development of these ducts.

Germinal epithelium degeneration and inflammatory and compensatory responses from prochloraz insult in the testis are chronic and sub-acute effects that might be caused by antagonism of the androgen-mediated phase of spermatogenesis (a late stage of testis morphogenesis); in both sexes, Müllerian ducts exhibited feminized reproductive development (Haselman *et al.*, 2018).

5. Discussion

Classification and terminology is often dissimilar in describing stages in anatomical/morphological/histological features or endpoints (e.g. Harczewic et al 2017). Although some authors attempted to answer some questions and harmonize definitions (e.g. De Rooij & Russell, 2000; Cevasco *et al.*, 2008), this could be the first obstacle for a microscopist.

It should be extremely important to harmonize results across laboratories regarding terminology of processes such as prespermatogenesis and early spermatogenesis classification (Harczkiewicz *et al.*, 2017), because assimilation to the most used anuran models (e.g. *Xenopus* sp.) is necessary for several scientific fields.

5.1 Histological endpoints used to measure effects of anti-androgenic chemicals

Hereinafter are listed histological endpoint (from the most to the less employed) used to measure effects of anti-androgenic compounds (see section 4.1), divided for each organ (see Section 2), regardless materials (e.g. anuran models, EDCs), methods (e.g. paraffin or resin embedded) and kind of result (e.g. scored results, descriptive results):

I. Testes
    1. Number of spermatogonia/cell nests/germ cells per seminiferous tubule (Cevasco *et al.*, 2008; Knechtges *et al.*, 2007; Orton *et al.*, 2018; Haselman *et al.*, 2018)
    2. Thickening of interlobular walls by hypertrophy/hyperplasia/fibrosis/infiltration (Cevasco *et al.*, 2008; Knechtges *et al.*, 2007; Haselman *et al.*, 2018)
    3. Seminiferous tubule diameters (Cevasco *et al.*, 2008; Orton *et al.*, 2018)
    4. Presence of ovotestis (Cevasco *et al.*, 2008; Knechtges *et al.*, 2007)
    5. Number of Sertoli cells (Cevasco *et al.*, 2008)
II. Ovary
    1. Presence/absence of diplotenic (postvitellogenic) oocytes (Orton *et al.*, 2018)
III. Reproductive ducts
    1. Presence/absence of Müllerian duct regression (Haselman *et al.*, 2018)
IV. Nuptial pads (NPs)
    1. Epithelium size/height (Orton *et al.*, 2018; van Wyk *et al.*, 2003)
    2. Number and volume of breeding (specialized mucous) glands (Orton *et al.*, 2018; van Wyk *et al.*, 2003)
    3. Number and keratinization of hooks (papillae – Orton *et al.*, 2018; van Wyk *et al.*, 2003)
    4. Darkness aspect (Orton *et al.*, 2018)
V. Larynx
    1. Number/size of dilator laryngis muscle fibres (Sassoon & Kelley, 1986)
VI. Miscellaneous
    1. Cytopathological alterations (Cevasco *et al.*, 2008; van Wyk *et al.*, 2003, Haselman *et al.*, 2018)

5.2 Sensitivity and Simplicity of histological endpoints for antiandrogenic compounds

Sensitivity measure the proportion/probability to detect a real positive results. Along with specificity influence the accuracy of an investigation. Basic histology and histopathology in medical and biological science have very wide range of sensitivity depending above all on the specific target (e.g. Adisa *et al.*, 2010). On the other hand, the simplest result seems to be the most uniform. In this section will be provided empirical consideration that followed the composition of this review. It should be possible to achieve similar insights by reading this manuscript; the author has intentionally explored basilar topics in order to reach the best of (his) knowledge.

Scoring descriptive results usually help the harmonization of data and interpretation of results. The different histological descriptive results in the reviewed literature adopted a grading system varying from 1 to 3 (spermatogenesis – Orton *et al.*, 2018), from 1 to 4 (percent of altered tissue – Halseman *et al.*, 2018), or they did not adopt (Cevasco *et al.*, 2008). Nonetheless, I would always recommend to describe specific histological abnormalities in order to permit a more entire view and comprehension of results.

Whenever results are not harmonized by standardization, their sensitivity and (usable) quality depletes regardless the quantity of data obtained. Countable and calculable endpoints easily address this limit.

According to this, histological endpoints as seminiferous tubule diameters, NPs epithelium size/height, number and volume of breeding (specialized mucous) glands of NPs, number and keratinization of hooks (papillae) of nuptial pads and number/size of dilator laryngis muscle fibres are easy calculable features with right available materials. Furthermore, they should be very simple when you are using the correct methods and you have basic knowledge of histology of target structures.

Countable endpoints should also be easy methods: it is just matter of absence or presence as for diplotenic (postvitellogenic) oocytes, Müllerian duct regression and Sertoli cells. The limit of the first mentioned might be a lower sensitivity since we are sampling exposed females that cannot manifest specific demasculinization, hence the presence of diplotenic oocytes could not be caused by an anti-androgenic compound if inherent. The limitation of Müllerian duct regression is probably the arduous detection and identification of the organ (giving that there is regression), but when found the absence of presence it is a sensitive result. Instead, the problem with Sertoli cells is that could be difficult to recognise and their absence/presence could be not sensitive and/or underrepresented: unlike in mammals, these cells are in amphibians are seasonal and their number depend on spermiation giving that they are detaching from the tubules and degenerating afterwards (see Section 2.1.2)

It is no coincidence that the most used endpoint is also the one employed with most heterogeneous methods: it is the estimation of spermatogonia/cell nests/germ cells per seminiferous tubule. Perhaps, it might be the most sensitive method (correlation between increase in spermatogonia/germ cells and decrease fertility) if a good scoring system is applied: the author suggests the one of Orton *et alii* (2018 – 3 stages: S.1 – primary spermatogonia, no tubules; S. 2 – early secondary spermatogonia; S. 3 –late secondary spermatogonia with or without tubules; See Fig. 12, 13) since it shows homogenous results and it seems the most straightforward to use.

The darkness aspect and colour of NPs could be a very simple method, but it is not easily standardisable since a lot of variables may occur. Nevertheless, is always worth to analyse alongside other above-mentioned NPs histological endpoints.

The less sensitive histological endpoints should be the ones that are found in both control and exposed groups or occasionally retrieved as testicular oocytes or ovotestes (it was also noticed with estrogen-like compounds: see Section 4 and EFSA, 2018) and thickening of interlobular walls by hypertrophy/hyperplasia/fibrosis/infiltration if not scored and homogenized.

As previously mentioned, when results are too descriptive, as cytopathological alterations, their sensitivity and (usable) quality wastes regardless the quantity of data obtained. For example, Haselman and collegues (2018) described a lot of cytological features (e.g. ovarian hypoplasia infiltrations, germinal epithelial thinning/loss with or without apoptosis or necrosis, mononuclear cellular infiltrate) that they could not use for statistical analyses. Nonetheless, this kind of approach might be the best to research across several differential diagnosis in order to program next step (i.e. preliminary studies), since every detail could be needed. On the other hand, a standardization of cytopathological endpoints or the support of a specialized veterinary pathologist is always desirable and appropriate.

It is obvious that other limits to sensitivity and simplicity are the difference between species, which yield our purpose more difficult and interesting/stimulating at the same time.

6. Conclusion

It should be fundamental to uniform classification and stages of reproductive germ cells and, consequently, histological endpoint scores in order to make possible any comparison of results from different studies regarding the same topic or the same class of compounds.

Most investigated organs for suborganismal histological endpoints for reproductive toxicity were worthily gonads, especially because their impairment would progressively lead to reproductive inability. Nevertheless, other primary or secondary reproductive organs should not be overlooked (e.g. external oblique muscle), since on the long run they could pose elusive and undiscernible risks as behavioural problem and fitness decrease in wild unmonitored populations (see Hayes *et al.*, 2010b). Moreover, it is always desirable the more frequent use of in vitro technologies (EFSA, 2018) but, whenever an in vivo investigation is inevitable, more information/results must be obtained from as many available organs as possible in order to respect the life of the sacrificed animals, in the best human manner.

Epigenetic, physical, pathogenic and environmental stressors and factors could unexpectedly affect the sensitivity of histological endpoints, hence they should be always evaluated, also for increasingly practice microenvironmental studies simulating natural conditions and necessary to define specific protection goals.

There are a lot of limitations and variables in ecotoxicological studies, but building a network, as Cost Action PERIAMAR is doing, will enhance the possibilities to safeguard against sub-lethal role of pesticides and conserve wild batracofauna and herpetofauna.

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