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Molecular and histological endpoints for developmental reproductive toxicity in *Xenopus tropicalis*: Levonorgestrel perturbs *anti-Müllerian hormone* and *progesterone receptor* expression

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ABSTRACT

There is an increasing concern regarding the risks associated with developmental exposure to endocrine disrupting chemicals and the consequences for reproductive capability. The present study aimed to refine the *Xenopus* (*Silurana*) *tropicalis* test system for developmental reproductive toxicity by characterising molecular and histological features of sexual development, and to explore effects of exposure to the progestagen levonorgestrel (LNG). Larvae were exposed to LNG (0, 3, 30, 300 ng/L) over the first three weeks of development, encompassing the beginning of gonadal differentiation. mRNA levels of *amh* (*anti-Müllerian hormone*), *amhr2* (*amh receptor 2*), *ipgr* (*intracellular progesterone receptor*), *mpgr beta* (*membrane progesterone receptor beta*), and *cyp19a1* (*cytochrome p450 19a1*) were quantified in larvae and juveniles (4 weeks post-metamorphosis). Relative *cyp19a1* and *amh* expression was used as a molecular marker for phenotypic sex of larvae. Gonadal and Müllerian duct development were characterised histologically in juveniles. Compared to controls, LNG exposure increased the expression of *amh* and *ipgr* in male larvae. In juveniles, *mpgr beta* expression was increased in both sexes and *amhr2* expression was decreased in males, implying persistent effects of developmental progestagen exposure on *amh* and *pgr* expression signalling. No effects of LNG on the gonadal or Müllerian duct development were found, implying that the exposure window was not critical with regard to these endpoints. In juveniles, folliculogenesis had initiated and the Müllerian ducts were larger in females than in males. This new knowledge on sexual development in *X. tropicalis* is useful in the development of early life-stage endpoints for developmental reproductive toxicity.

1. Introduction

There is an increasing concern regarding the risk posed by endocrine disrupting chemicals to the developing endocrine and reproductive systems. Early life chemical perturbation of the development of reproductive organs including the gonads and the Müllerian ducts has been shown to cause reproductive failure later in life in wildlife species as well as in humans (Goyal et al., 2003; Hill and Janz, 2003; Blomqvist et al., 2006; Pettersson et al., 2006; Crain et al., 2008; Gyllenhammar et al., 2009; Kvarnryd et al., 2011). The Müllerian ducts are precursors of the female reproductive tract and they are present in both sexes during early life stages of vertebrates (except teleost fish). In female mammals, they develop into oviducts, uterus, cervix and vagina whereas in female birds, reptiles and frogs they develop into oviducts (Adkins-Regan, 1987). Evidence suggests that female reproductive disorders observed in wildlife and humans may be symptoms of incorrect differentiation of the ovary and the embryonic Müllerian ducts due to endocrine disruption during early life stages (Crain et al., 2008). However, causal relationships between the female reproductive disorders in humans and wildlife and exposure to environmental chemicals remain to be elucidated (UNEP/WHO, 2013). To determine relationships between chemical exposure and developmental disorders in Müllerian duct-derived tissues appropriate test systems need to be developed.

Research on endocrine disrupting effects of chemicals has thus far focused mainly on perturbation of estrogen, androgen and thyroid signalling pathways. The knowledge on chemical effects on progesterone signalling, which is a key regulatory pathway in sexual development and reproductive function, is far less developed. However, recent research has highlighted progestagens (here defined as synthetic or natural progesterone) as potent endocrine disrupting chemicals in the aquatic environment (Arnold et al., 2014; Säfholm et al, 2014; Svensson et al, 2014). Progestagens are widely used in human and

Abbreviations: Amh, anti-Müllerian hormone; amhr2, anti-Müllerian hormone receptor 2; cyp19a1, cytochrome p450 19a1; LNG, levonorgestrel, NF, Nieuwkoop and Faber; ipgr, intracellular progesterone receptor; mpgr, membrane progesterone receptor; *X. tropicalis*, *Xenopus tropicalis*.

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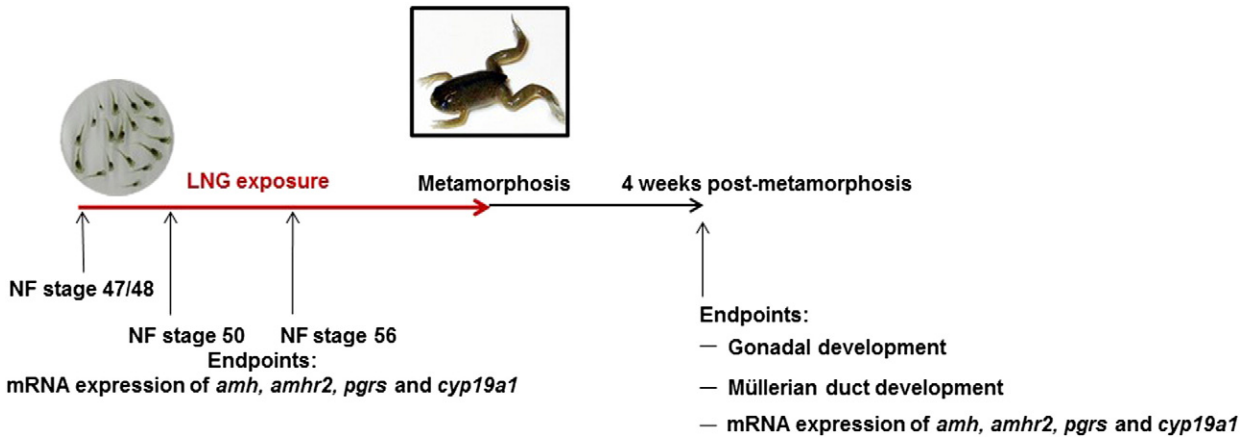

Fig. 1. Experimental design with regard to levonorgestrel (LNG) exposure period, sampling time points, and endpoints during larval and juvenile stages of *Xenopus tropicalis*. NF stage = Nieuwkoop and Faber stage (Nieuwkoop and Faber, 1956). *amh* (*anti-Müllerian hormone*), *amhr2* (*anti-Müllerian hormone receptor 2*), *pgrs* (*progesterone receptors*), and *cyp19a1* (*cytochrome p450 19 a1*).

veterinary medicine (e.g. in contraceptive pills) and there are more than 20 types of progestagens on the global market (according to The American National Institutes of Health). They are constantly released into the environment from sewage treatment plants, agricultural areas via farm animal waste, pharmaceutical industries, and wastewater irrigation. A number of progestagens including levonorgestrel (LNG) have been detected in lakes, rivers, and streams, which may result in exposure of the aquatic wildlife to concentrations ranging from one to a few tens of ng/L (Petrovic et al., 2002; Vulliet et al., 2008; Al-Odaini et al., 2010; Vulliet and Cren-Olive, 2011).

Progestagens are potent developmental reproductive toxicants targeting Müllerian duct differentiation in amphibians, birds and mammals, and gonadal development in fish, amphibians, and mammals (Chen et al., 2007; Gray et al., 2001; reviewed in Hayes, 1998; Kvarnryd et al., 2011; Liang et al., 2015; Lorenz et al., 2011; Stoll et al., 1990). The underlying mechanisms for progestagen-induced developmental reproductive toxicity are not fully understood at present. However, it is known that these compounds have a higher affinity to the progesterone receptor (Pgr) than progesterone itself. For instance, the affinity of LNG to the human Pgr is 150–323% compared to that of progesterone (Kumar et al., 2015). As proper Pgr expression is crucial for normal uterine development from the Müllerian ducts (Lydon et al., 1995) disrupted Pgr expression during critical developmental windows is a potential initiating mechanism involved in LNG-induced developmental toxicity. In amphibians, larval LNG exposure resulted in a lack of oviducts and sterility in adult females which might imply Müllerian duct dysgenesis (Kvarnryd et al., 2011). In *Xenopus tropicalis*, *ipgr* (*intracellular progesterone receptor*), and *mpgr beta* (*membrane progesterone receptor beta*) are expressed during larval development (Jansson et al., 2015), making them potential targets for progestagen action.

The anti-Müllerian hormone (Amh) is required for proper gonadal and Müllerian duct differentiation in vertebrates (Behringer et al., 1994; Josso et al., 2013). In mammals, Amh-deficiency results in inhibited Müllerian duct regression in males and in gonadal abnormalities in both sexes (Behringer et al., 1994; Josso et al., 2013). Embryonic over-expression of Amh resulted in inhibition of Müllerian duct differentiation and masculinisation of the ovaries in mice (Behringer et al., 1990). Furthermore, similar to progesterone, Amh is involved in oogenesis (Nilsson et al., 2011). Given the key role of Amh in sexual development and function, the expressions of *amh* and its receptor 2 (*amhr2*) are interesting to explore as potential targets for developmental reproductive toxicants and as early life molecular markers for developmental reproductive toxicity.

X. tropicalis represents an excellent model for investigating developmental reproductive toxicity for several reasons (Berg et al., 2009). The organisation and components of the amphibian hypothalamus–pituitary–gonadal axis are very similar to those in higher vertebrates (reviewed in Kloas and Lutz, 2006). The genome of *X. tropicalis* is sequenced (Hellsten et al., 2010), facilitating gene expression analysis. Being water-dwelling throughout life *X. tropicalis* is a suitable model in experimental aquatic toxicology. It allows a more comprehensive and detailed analysis of reproductive organ development compared with commonly used teleost fish models that lack Müllerian ducts. The differentiation of the Müllerian ducts and gonads in *X. tropicalis* is very sensitive to endocrine disruption (Pettersson et al., 2006; Gyllenhammar et al, 2009; Porter et al., 2011). *X. tropicalis* has a generation time of about 4–6 months which provides unique possibilities for life-cycle studies compared to other frog species. Life-cycle studies in *X. tropicalis* have revealed that exposure to endocrine disrupting chemicals during sex differentiation can result in reproductive failure in the adult frog (Pettersson et al., 2006; Gyllenhammar et al., 2009; Kvarnryd et al., 2011). However, as life-cycle studies are very time- and resource consuming the characterisation of sensitive early life-stage endpoints/biomarkers for developmental reproductive toxicity would increase the usefulness of the *X. tropicalis* test system.

Currently there is no general marker of genetic sex available for *X. tropicalis*. Molecular tools to determine phenotypic sex before the gonads are morphologically different therefore need to be developed. *Cytochrome p450 19a1* (*cyp19a1* or *aromatase*, catalysing the biosynthesis of estrogens from androgens) has been proposed as a female marker as the expression in the ovary is higher than that of the testis during sex differentiation (Duarte-Guterman and Trudeau, 2011; Navarro-Martín

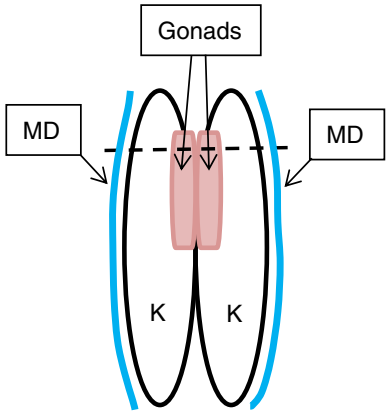

Fig. 2. Schematic illustration of the localisation of the histological section (dotted line) through the urogenital complex of juvenile *Xenopus tropicalis*. MD = Müllerian duct. K = kidney.