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Research paper

Temperature- *vs.* estrogen-induced sex determination in *Caiman latirostris* embryos: Both females, but with different expression patterns of key molecules involved in ovarian development

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ABSTRACT

Caiman latirostris is a species with temperature dependent sex determination (TSD), which implies that the incubation temperature of the eggs is the main factor that determines the sex during a thermosensitive period (TSP). However, estrogens play a critical role in this process. The administration of 17β-estradiol (E_2) previous to TSP overrides the effects of male incubation temperature, producing phenotypic females. This effect has been defined as sex reversal or estrogen-induced sex determination (E_2SD). The aim of the present study is to describe similarities and differences in the effects of TSD and E_2SD treatment conditions on ovary development. Our results show that the two treatment conditions studied are able to produce different ovaries. Treatment with E_2 modified the expression pattern of estrogen receptor alpha and progesterone receptor, and expression of the enzyme aromatase. Moreover, in E_2SD females, the proliferation/apoptosis dynamic was also altered and high expression of TAp63 was observed suggesting the presence of greater DNA damage in germ cells. To the best of our knowledge, this is the first report that describes the morphology of the female gonad of *C. latirostris* in three stages of embryonic development and shows the expression of TAp63 during the gonad development of a reptile. It is important to emphasize that the changes demonstrated in E_2SD female gonads of embryos show that environmental compounds with proven estrogenic activity alter the follicular dynamics of *C. latirostris* in neonatal as much as in juvenile animals, endangering their reproductive health and possibly bringing consequences to ecology and evolution.

1. Introduction

After oocyte fertilization the sex of the offspring of all crocodilian and many turtle species is determined by the environment. The incubation temperature of the eggs during a critical period of development (thermosensitive period-TSP-) is the main factor that determines the sex of the progeny in the absence of sex chromosomes (Valenzuela et al., 2014). This process is known as temperature dependent sex determination (TSD) (Gilbert, 2000; Lang and Andrews, 1994). TSD initiates a cascade of molecular events that favour the development of one sex or the other altering the control of gene expression and cellular signaling by steroid hormones, hormone receptors and steroidogenic enzymes (Mizoguchi and Valenzuela, 2016). Some molecular events could precede the formation of the histological architecture that characterizes a testis or an ovary (Rhen and Schroeder, 2010).

Abbreviations: C. latirostris, *Caiman latirostris*; DAB, diaminobenzidine; E_2, 17β-estradiol; E_2SD, estrogen-induced sex determination; EDCs, endocrine-disrupting compounds; ESRs, nuclear estrogens receptor; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; GAM, gonadal-adrenal-mesonephros; IHC, immunohistochemistry.; IOD, integrated optical density; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor; TAp63, isoform TA from p63 tumor suppressor; TSD, temperature-dependent sex determination; TSP, thermo-sensitive period; TUNEL, terminal deoxynucleotidyl transferase-mediated sUTP nick end-labelling; VASA, ATP-dependent RNA helicase.

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Estrogens play a critical role in sex determination in crocodilians and turtles. The administration of 17β-estradiol during TSP overrides the effects of male incubation temperature, producing phenotypic females in *T. scripta* and *A. mississippiensis* among others (Crain et al., 1997; Crews et al., 1996; Milnes et al., 2002). This effect has been defined as sex reversal or estrogen-induced sex determination (E_2SD) (Crews et al., 1991; Holleley et al., 2016; Tousignant and Crews, 1994; Wibbels et al., 1991, 1992). Moreover, the administration of 17β-estradiol during TSP has been proposed as an alternative way to improve the recovery of endangered reptile species, by skewing the population sex ratio to one that favours reproductive females (Crews and Wibbels, 1993).

TSD has been demonstrated in *Caiman latirostris* (Crocodylia: Alligatoridae) following a female-male-female pattern, since eggs incubated at 30–31 °C produced 100% females, 32 °C produced approximately 70% females, and incubation at 33–34 °C produced only males. The transitional range of temperature was >31° to <33 °C for female to male transition, and >34 °C to <34.5 °C for male to female transition (Parachú Marcó et al., 2017; Pina et al., 2003; Stoker et al., 2003). Sex reversal or E_2SD has also been described in this species (Pina et al., 2003; Stoker et al., 2003).

There is a general agreement that in the determination of the female sex the application of estrogens is the physiological equivalent of the incubation temperature (Crews et al., 1996). Estrogens act primarily through their specific receptors and receptor expression levels may reflect the sensitivity of an organ/tissue to these steroids. Estrogens are involved in gonad differentiation, maturation of the female reproductive tract and mating behaviour (Iguchi et al., 2006; McLachlan, 2001).

Cytochrome P450 aromatase (aromatase) is the enzyme responsible for the conversion of androgens to estrogens, and as such it plays a key role in many biological functions that are estrogen-dependent, including sex differentiation of vertebrate developing embryos (Place and Lance, 2004; Yao and Capel, 2005). Treating eggs with an aromatase inhibitor to block estrogen synthesis inhibits ovarian development in alligator embryos incubated at female-producing temperature. In spite of that, the gonad is not totally masculinized (Lance and Bogart, 1992). In turtles (Dorizzi et al., 1994) and birds (Elbrecht and Smith, 1992), however, complete masculinization of the gonad has been achieved by treating eggs with an aromatase inhibitor (Rhen and Lang, 1994; Wibbels and Crews, 1994). These data indicate that estrogen would be necessary for female gonadal development in these species and that, in the absence of estrogen, testis would develop (Gabriel et al., 2001). Several studies performed on the whole gonad/adrenal/mesonephros (GAM) complex were unable to find differences between male- and female-producing temperatures in endogenous estrogen content, aromatase activity or aromatase gene expression during the thermosensitive period for sex determination (Gabriel et al., 2001; Milnes et al., 2002; Smith et al., 1995; Smith and Joss, 1994). Thus, the key role of aromatase and estrogens in the first steps of ovarian differentiation has been questioned, and extragonadal organs or tissues, such as adrenal, mesonephros, brain or yolk, are considered possible targets of temperature and sources of the estrogens acting on gonadal sex differentiation. Nevertheless, experiments and assays carried out on isolated gonads (separated from the adrenal/mesonephros), provide evidence that the gonads themselves respond to temperature shifts by modifying their sexual differentiation and are the site of aromatase activity and estrogen synthesis during the thermosensitive period (Pieau and Dorizzi, 2004). Works performed in *Xenopus laevis* showed that the oocyte participates significantly in the synthesis of ovarian estrogens and this may be a common feature of vitellogenic vertebrates (Gohin et al., 2011).

As is well known, the processes of proliferation and apoptosis have been closely related to sex determination and gonadal differentiation. Ovarian differentiation in alligators is characterized by proliferation of germ cells in the cortex of the gonad (Smith and Joss, 1993), while apoptosis of germ cells is related to testis differentiation in some species of fish (Uchida et al., 2002; Yamamoto et al., 2013). Additionally, it remains well accepted that significant germ cell apoptosis and oocyte loss during the establishment of the primordial follicle pool in most female mammals (Sun et al., 2017). TAp63 is an isoform of p63 that belongs to the p53 family of transcription factors. This isoform, is the only p53 family member identified so far that participates in the oocyte DNA damage response in mammals. TAp63 had been proposed to be the "guardian of meiotic recombination," driving to apoptosis any oocytes that fail to rejoin their chromosome arms on time (Carroll and Marangos, 2013). This protein induces cell cycle arrest and initiates DNA repair or apoptosis in mouse after the chromosome crossing-over process (Petre-Lazar et al., 2007). It is generally accepted that gonadal apoptosis is a hormonally regulated process. Estrogens are potential regulators of gonadal apoptosis during sex differentiation in fishes (Uchida et al., 2002; Yamamoto et al., 2013). To our knowledge, the presence of p63 has never before been described in reptiles. Humans and wildlife are exposed to numerous hormonally active pollutants commonly referred to as endocrine disruptor chemicals (EDCs) (Committee on Hormonally Active Agents in the Environment, 1999). The majority of known EDCs exhibit estrogenic activity and are classified as xenoestrogens (Sonnenschein and Soto, 1998).

Estrogens actions are mediated by a plethora of molecular mechanisms that constitute different pathways through which EDCs trigger their effects. Female and male caiman reproductive tissues are highly sensitive to the effects of EDCs such as endosulfan, atrazine and bisphenol A (Durando et al., 2016, 2013; Rey et al., 2009; Stoker et al., 2008, 2003). Prenatal exposure to estrogens or EDCs modifies ovarian follicular dynamics and hormonal steroid levels in postnatal female caimans (Stoker et al., 2008). Other results revealed that early postnatal exposure to EDCs alters the temporal and spatial expression pattern of histofunctional differentiation biomarkers in the oviduct later in life (Galoppo et al., 2016). Since *C. latirostris* can be naturally exposed to EDCs (Stoker et al., 2011, 2013), the search for knowledge about estrogen's mechanism of action on this species is of particular interest both to assess the impact of EDCs on caiman populations and to better characterize *C. latirostris* as a biomonitor of ecosystem health.

The aim of the present study is to expand the knowledge regarding the developmental patho-physiology of the embryonic reproductive system of *C. latirostris*, describing similarities and differences in the effects of TSD and E_2SD conditions on ovary development. Previous results of our group found an alteration in follicular dynamics in neonatal and juvenile E_2SD females (Stoker et al., 2008). This leads us to propose the hypothesis that these females could show alterations in key molecules during gonadal development, already in the embryonic stages. We analyzed the protein expression of the estrogens and progesterone receptors, as well as the expression of the enzyme aromatase and cell proliferation, DNA damage and apoptosis in both TSD and E_2SD females in order to identify possible targets of EDC actions.

2. Materials and methods

2.1. Animals

All laboratory and field work was conducted according to the published guidelines for the use of live amphibians and reptiles in field and laboratory research (American Society of Ichthyologists and Herpetologists, 2004) and in full compliance

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