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Tilapia male urinary pheromone stimulates female reproductive axis

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ABSTRACT

Mozambique tilapia males congregate in leks where they establish dominance hierarchies and attract females to spawn in sandy pits. Dominant males store more urine than subordinates and the pattern of urination and the high sensitivity of females to male urine suggest chemical signalling via the urine. Here we show that pre-ovulated and post-spawn females when exposed to dominant male urine increased significantly, in less than 1 h, the release rate of the maturation-inducing steroid 17,20β-dihydroxypregn-4-en-3-one which is maintained elevated for at least 6 h. This indicates a pheromonal role for male urine in the synchronisation of spawning. Furthermore, we show that the lack of affinity of 17,20βP to sex steroid binding globulin explains, at least partly, its rapid release and lack of detection in the blood. Thus tilapia urine involvement in several communication processes confirms that cichlids have evolved a sophisticated chemical signalling system together with their complex visual, acoustic and behavioural displays.

1. Introduction

The use of waterborne chemical signals to control physiological process and behaviour has been observed in several fish (Chung-Davidson et al., 2011; Hara, 1994; Rosenthal and Lobel, 2005; Stacey and Sorensen, 2005). Among this group of chemicals, sex pheromones are involved in reproduction by mediating location of suitable partners, evoking appropriate behavioural and endocrine responses, and improving synchronisation of gametogenesis, spawning, fertility and paternity (see reviews by Burnard et al., 2008; Stacey et al., 2003). The identification and characterisation of these compounds is important for understanding fish reproductive physiology (Stacey, 2011) and as a potential tool for population management (e.g., aquaculture and species invasions) (Johnson and Li, 2010; Sorensen and Stacey, 2004).

Pheromonal responses can occur at different levels, scales and contexts. Behavioural responses to a given stimulus occur usually within seconds, (Liley et al., 1986; Rouger and Liley, 1993; Stacey et al., 1989) but relatively rapid physiological changes, e.g. sex steroid synthesis and metabolism, are also possible within minutes or hours (Bayunova et al., 2011; Dulka et al., 1987; Scott et al., 1994).

Sex steroids are produced by the gonads through gonadotrophic stimulation and released into the blood to be transported to target organs. Therefore, steroid production is traditionally determined by analysis of blood samples in vertebrates. However, handling and sampling induce acute stress responses that may mask any response (Scott and Ellis, 2007; Scott et al., 2008). Consequently, there is increasing interest in the use of non-invasive procedures, including determination of steroid content in water and faeces, in fishes (Scott et al., 2008). The fish gill is considered to be the main route for release of free steroids at a rate that reflects largely their plasma concentrations. Indeed, several studies suggest that free steroids are preferentially released via the gills and are found at much lower concentrations in the urine or faeces compared to conjugated metabolites (Ellis et al., 2005; Miguel-Queralt and Hammond, 2008; Scott et al., 2008; Siefkes et al., 2003; Vermeirssen and Scott, 1996). Therefore, changes of steroid concentration in water samples can parallel those in blood, taking into account the dilution effect, and estimation of sex steroids in the water is a reliable indicator of fish endocrine status (Scott and Sorensen, 1994; Scott et al., 2008; Sebire et al., 2009). In cases of lack of correlation between blood plasma concentrations and release rates of steroids this has been ascribed to several factors including steroid characteristics and metabolism, and the presence of steroid binding globulins in blood with differing affinity for steroids (Scott and Sorensen, 1994; Scott et al., 2008).

The Mozambique tilapia (*Oreochromis mossambicus*; hereafter 'tilapia') is a polygynous maternal mouth-brooding African cichlid. In nature, males aggregate in breeding arenas (leks) and dominant males defend small territories centred on pits (nests) that they dig in the sand, adopting a typical black colouration; visiting females spawn in the territory of a dominant male and then move away from the males to mouth brood the eggs (Almeida et al., 2005; Barata et al., 2007; Oliveira and Almada, 1998; Russell et al.,

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2012; Turner, 1986). Furthermore, signalling of male dominance via controlled urination has been demonstrated. Unlike subordinate males and females, dominant tilapia males store urine which is a vehicle for potent odorants actively released during aggressive disputes and mating behaviour (Almeida et al., 2005; Barata et al., 2007, 2008; Miranda et al., 2005). Moreover, the urinary bladders of dominant males are larger and more muscular than those of subordinate males or females, suggesting an adaptation facilitating storage of larger urine volumes for longer and more frequent urination in the appropriate social context, which may modulate aggression between opponent males (Keller-Costa et al., 2012). Males can discriminate the sexual status of females using olfactory cues (Miranda et al., 2005) and when in the presence of ovulated females, their urination rate increases (Almeida et al., 2005; Barata et al., 2008). The olfactory potency of male urine depends on the social status of the donor (Barata et al., 2007). These observations indicate that tilapia males release a pheromone via the urine which influences female spawning (Barata et al., 2008). However, the physiological and/or behavioural effect of the male pheromone on females is still unknown.

To determine the possible priming effect of the sex pheromonal compound present in male urine on female tilapia we measured their response, in terms of sex steroids, to male urine. We chose to measure steroids secreted mainly during the secondary growth phase (testosterone and 17β-estradiol; E_2) and during final maturation of the oocytes (17,20β-P). Testosterone is produced by the theca cells surrounding the follicle under gonadotrophic stimulation and has positive feedback effects in the pituitary (Nagahama et al., 1995). Testosterone is the precursor of E_2 produced in the granulosa cells, which promotes ovarian growth through the stimulation of synthesis and secretion of vitellogenin and egg shell proteins in the liver (Lubzens et al., 2010; Nagahama et al., 1995; Senthilkumaran et al., 2004; Young et al., 2005). After oocyte growth, a shift in steroidogenesis leads to the production of 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) also in the granulosa cells which induces the resumption of meiosis and final oocyte maturation (Nagahama, 1997; Senthilkumaran et al., 2004). In goldfish 17,20β-P is also released to the water and is perceived by males thereby stimulating the endocrine system, spermiation as well as increased fertility and paternity (Dulka et al., 1987; Scott and Sorensen, 1994; Zheng et al., 1997).

Here we establish that urine from tilapia males contains a pheromone that primes the female's reproductive system by increasing the production and release rates of the maturation-inducing steroid 17,20β-P. We also show that these changes can only be detected in the water, possibly because the tilapia plasma sex steroid binding globulin has low affinity for 17,20β-P.

2. Materials and methods

2.1. Experimental animals

Adult Mozambique tilapia (*O. mossambicus*) of both sexes were taken from a stock population established at the Centre of Marine Sciences, University of the Algarve. Fish were tagged and 8 groups of one male and four females were setup in tanks of 250 L with sand substratum and kept at 27 ± 1 °C under a photoperiod of 12 h light/12 h dark (lights on at 7.30 a.m.) and fed twice daily. Spawning occurred spontaneously in each tank, producing viable offspring. After each spawning, as soon as the females were seen carrying the eggs (usually within less than a day), they were taken from their mouth to maintain the females' ovulatory cycle and predict the next ovulation (Miranda et al., 2005). Twice a week, urine samples were taken from each of the 8 males, pooled with equal volumes of urine from each male and frozen (−20 °C) as previously described (Keller-Costa et al., 2012). Fish care and experimentation complied with the guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals under a "Group-1" license issued by the veterinary directorate "Direcção Geral de Veterinária" of the Ministry of Agriculture, Rural Development and Fisheries of Portugal.

2.2. Exposure of females to male urine

Pre-ovulatory and post-spawning females were exposed to male urine and were expected to have different endocrine response as they represent different gonadal states. Only females with a regular ovulation cycle were used. A female was determined as regular if 3 consecutive ovulation cycles were of the same length (±2 days). Females were pre-ovulatory two days prior to the predicted date of ovulation and post-spawn three days after the last ovulation. Ovulation cycle length at 25 °C of regular females is around 15 days (between 9 and 19 days) (Miranda et al., 2005). Experimental females, average weight 64.84 g ± 1.63, were placed in a glass isolation tank overnight and on the next day (i.e., one day prior to ovulation, or four days after spawning, respectively), each female was placed in an identical tank with a volume of clean water (de-chlorinated tap-water) proportional to the weight of the fish 10 g L^{-1}; 6–8.5 L, (Scott et al., 2008). After 1 h, 1 L of water was taken (by siphoning with a tube previously placed in the tank) for extraction (see below) and replaced with clean water. A volume of the pooled male urine was added to the tank to give a dilution 1:10,000 of urine: water. One hour later, another litre of water was taken for extraction and replaced with another litre of clean water containing male urine (at 1:10,000). This process was repeated 2, 4 and 6 h after the initial addition of the male urine. At the end of the experiment urine and blood samples (from 6 to 8 females per group) were taken from each female under anaesthesia with MS222 (50 mg L^{-1} water). Control experiments were carried out exactly as described above except that the urine was replaced with distilled water. Another series of controls was carried out in exactly the same manner except that no fish was present (using 5 L water and male urine at 1:10,000) and no steroids were detected. Each female was used four times; as pre- and as post-ovulated, both in the presence and absence of male urine. The order of treatment was varied among females, but all were killed after the final exposure and replaced in the family tanks by new females. At least one ovulation cycle was allowed between successive treatments, when females were returned to their original family tanks. Total steroids (free plus sulphate and glucuronide) in the pool of male urine was; 24.5 ng ml^{-1} for 17,20βP, 14.3 ng ml^{-1} for cortisol, 34.1 ng ml^{-1} for testosterone and undetectable for E_2.

2.3. Steroid analysis

Blood plasma and urine (100 μl) were mixed with 100 μl of distilled water and extracted twice with 4 ml of diethyl ether to obtain free steroids. Water samples were extracted using 'Isolute' C18 500 mg solid-phase extraction cartridges (International Sorbent Technology Ltd., Hengoed, UK), under vacuum (flow rate approximately 4 ml min^{-1}), according to the manufacturer's instructions. Methanol (10 ml) was used to activate the cartridges and to elute the steroids.

Extracts were dried under nitrogen, reconstituted in 0.5 M phosphate–gelatine buffer, pH 7.6 and steroids measured by radioimmunoassay (RIA): E_2 (Guerreiro et al., 2002) and 17,20βP (Canario et al., 1989) and testosterone. The testosterone antiserum was kindly donated by Dr. A.P. Scott and had the following cross-reactions: 63% for androstenedione, 35% for 11-ketotestosterone, 55% for 11β-hydroxytestosterone, 40% for 5α-androstan-17β-ol-3-one, 31% for 5β-androstan-17β-ol-3-one, 12% for 5β-androstane-3α,17β-diol, 25% for 5α-androstane-3α,17β-diol. The