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Thermosensitivity of the sex differentiation process in the African catfish, *Clarias gariepinus*: Determination of the thermosensitive period

Saïdou Santi [a,b,*], Vincent Gennotte [a], Aboubacar Toguyeni [b], Charles Mélard [a], Nadine Antoine [c], Carole Rougeot [a]

[a] Aquaculture Research and Education Center (CEFRA), University of Liège, 10 Chemin de la Justice, B-4500 Tihange, Belgium
[b] Natural Resources and Environmental Sciences Research and Studies Laboratory (LERNSE), Institute of Rural Development, Polytechnic University of Bobo-Dioulasso, Bobo-Dioulasso, Burkina Faso
[c] FARAH, Department of Morphology and Pathology, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium

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ABSTRACT

Gonadal sex differentiation in gonochoristic fish is generally labile and under the control of two interacting processes: genetic sex determination (GSD) and environmental sex determination (ESD). Numerous experimental studies deal with temperature induced-sex differentiation in teleosts, but none focused on the African catfish *Clarias gariepinus*. The aim of this study was to confirm the thermosensitivity of the sex differentiation process and to determine the thermosensitive period during the African catfish development. Fish were exposed to high temperature (36 °C) for 3 days at different periods during ontogenesis. The treatment was applied every 3 days from fertilization until 29 days post-hatching (dph). Before and after the thermal treatment, fish were reared at 28 °C. Gonadal development was histologically characterized on fish sampled at 10, 15, 20, 25, 35, 45, 55 and 70 dph. Our results demonstrated that the African catfish displays a thermosensitivity of the sex differentiation process, with a masculinizing effect of high temperature (36 °C). The most thermosensitive period extended from 6 to 8 dph. Fish batches exposed to 36 °C during this period showed a sex-ratio skewed towards the male phenotype, ranging from 58 to 100% (high inter-familial variability). The African catfish gonads (male and female) stayed histologically undifferentiated until 20 dph. Obvious signs of gonadal differentiation clearly appeared at 25 dph in females and at 45 dph in males.
Variability in sex-ratios between progenies and in the response to thermal treatment suggests a role of minor genetic factors and interactions between genomic and environmental determinants in the expression of the sexual phenotype.
Statement of relevance:

1. This paper provides novel methods to control African catfish *Clarias gariepinus* sex differentiation through high temperature exposure and then to produce all-male populations.
2. Our work underlines the possibility to significantly reduce high temperature (masculinizing effect) treatment period to 3 days and consequently increase survival rate of progenies after treatment.
3. This study also shows the inter-family variability of thermosensitivity on the sex differentiation process in *Clarias gariepinus*.

1. Introduction

Sex determination and sex differentiation processes are widely investigated in fish (Baroiller et al., 1999; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Guerrero-Estèvez and Moreno-Mendoza, 2010). Sex determination can be defined as the genetic and/or environmental processes that influence the sex definition, whereas sex differentiation refers to gonadal development into ovary or testis, once sex was determined (Hayes, 1998; Devlin and Nagahama, 2002). According to Piferrer et al. (2012b), sex-ratio is the result of complex interactions between genetic, biochemical and environmental factors, finally resulting in the expression of a female or male phenotype at the individual level.

Among the 30,000 existing species (Nelson, 2006), fish display a large variety of sex determination mechanisms and patterns of sexual differentiation (Guerrero-Estèvez and Moreno-Mendoza, 2010). Hence, they

* Corresponding author at: Aquaculture Research and Education Center (CEFRA), University of Liège, 10 Chemin de la Justice, B-4500 Tihange, Belgium.
E-mail address: s.santi@doct.ulg.ac.be (S. Santi).

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are very good models for research and applied studies on vertebrate sex determination (Devlin and Nagahama, 2002). Two systems of sex determination were reported in gonochoristic fish. The genetic sex determination (GSD) includes monofactorial or polygenic systems in which sex is respectively determined by a gene located on a single chromosome or by the accumulative action of different genetic factors involved in its determination (Baroiller et al., 1999; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Guerrero-Estèvez and Moreno-Mendoza, 2010). In species with an environmental sex determination (ESD) system only, sex is determined after fertilization by environmental factors and there is no evidence of the presence of sex chromosomes (Ospina-Álvarez and Piferrer, 2008).

In species with ESD, temperature is the main environmental factor affecting the sex-ratio – temperature-dependent sex determination (TSD) – but an influence of pH or social factors was also reported in several species (Conover and Kynard, 1981; Baroiller et al., 1999; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002). Moreover, in some species with GSD, such as Nile tilapia (*Oreochromis niloticus*), environmental factors and particularly high temperatures (>34 °C) can act on the sex differentiation process and orientate the gonad phenotype, suggesting an interaction between genetic and environmental sex determination processes (Baroiller et al., 1995; Baroiller and Clota, 1998; Baroiller and D'Cotta, 2001; Bezault et al., 2007). On this basis, Piferrer et al. (2012b) concluded on the existence of a continuum between GSD and TSD systems with species in which sex is determined by sex chromosomes and is also influenced by temperature effects.

In Nile tilapia, it was shown that high temperatures act on the expression of the cytochrome P450 aromatase that catalyzes estrogen synthesis. Genetic males exhibit lower levels of aromatase gene expression in the gonad and lower 17β-estradiol plasma concentrations than females. Additionally, the aromatase enzyme activity is sexually dimorphic in the brain of Nile tilapia fry, with a high and low activity in future females and males respectively (D'Cotta et al., 2001). Administration of an aromatase inhibitor during the sex differentiation period caused a repression of estrogen synthesis and resulted in the production of phenotypic males in several fish species (Piferrer et al., 1994; Guiguen et al., 1999, 2010). Similarly, the exposure of genetic females to high temperatures during the period of gonad differentiation induced a repression of aromatase gene expression in the gonad, resulting in the expression of a male phenotype (D'Cotta et al., 2001; Strüssmann and Nakamura, 2002; Guiguen et al., 2010).

Since the first demonstration of the influence of temperature on the sex differentiation process in the Atlantic silverside *Menidia menidia* (Conover and Kynard, 1981), an important number of experimental studies focused on this topic (Ospina-Álvarez and Piferrer, 2008; Baroiller et al., 2009a, 2009b; Guerrero-Estèvez and Moreno-Mendoza, 2010; Piferrer et al., 2012b). However, to our knowledge, none concerned the African catfish, *Clarias gariepinus*. In this species, although a genetic sex determination system (XY/XX) was suspected by Viveiros et al. (2001), the process of sex differentiation remains unclear and the interaction between genotype and environment still needs to be explored.

Previous experiments performed in our laboratory identified a thermosensitive period for gonad differentiation in African catfish juveniles. Temperatures above 32 °C significantly skewed the sex-ratio (from 55 to 100% of males) when applied before 12 days post-hatching (dph) (Rougeot et al., 2008). Unlike other thermosensitive species in which long thermal treatments are required to control the sex differentiation process, our results also suggested that short treatment durations (3 days) are sufficient to induce a partial or total sex reversal of genetic females in a population of African catfishes.

In the African catfish, under laboratory conditions, the first histological differences in male and female gonads are visible at 28 dph. At this age, the formation of an ovarian cavity and the presence of germ cells with nuclei in meiotic prophase are observable in female gonads. Testes are morphologically recognizable from 42 dph. They are characterized by their crenated surface, the presence of primary spermatogonia and many Leydig cells in the stromal tissue (Van Den Hurk et al., 1989).

Based on these preliminary results, the aim of this study was to confirm the masculinizing effect of high temperature during the development of the African catfish, *C. gariepinus* and to precisely determine the thermosensitive period of the sex differentiation process. A histological study of the juvenile gonad development was performed to characterize the timing of the histological differentiation under our experimental conditions.

2. Materials and methods

The experiments were carried out according to the European animal welfare recommendations and to the guidelines of the University of Liège Ethical Committee.

2.1. Egg and larva production

Full-sib eggs and larvae were obtained by artificial reproduction of domesticated African catfish breeders reared in 4 m^2 tanks in a recirculating system at 27 °C and 12 L:12 D photoperiod and fed ad libitum. Mature females were selected based upon the morphology of the genital papilla and abdominal distension. In order to induce spawning, females received a single intra-muscular injection of 0.5 ml kg^{-1} body weight of Ovaprim® (Syndel, Canada). Eggs were stripped at 243 °C h^{-1} after hormonal injection and fertilized with intratesticular semen from males previously euthanized by an overdose (200 mg kg^{-1}) of benzocaïne (Sigma-Aldrich). Twenty-three different full-sib progenies (from 1 male and 1 female) were used in the experiments. After fertilization, eggs were transferred into 1.5 l Zug bottles for incubation until hatching (24 h at 28 °C).

2.2. Larval and juvenile rearing

Two day post-hatching larvae were transferred into 50 l aquaria at an initial stocking density of 10 larvae l^{-1}. Aquaria were supplied with water from a recirculating system at 28 °C. From 3 dph, fish were fed with a commercial diet (Gemma Micro, Skretting, 54% proteins, 14% lipids for 17 days; from 21 dph: Nutra, Skretting, 45% proteins, 12% lipids for 49 days). Feeding was performed 5 times a day ad libitum. At 40 dph, survival rate was calculated by counting all the fish in each aquarium. The number of fish was reduced to 200 per aquarium in order to maintain an optimal biomass and a good water quality (3.8 ± 2.9 mg $[N\text{-}NH_4^+]$ l^{-1} and 1.0 ± 0.6 mg $[N\text{-}NO_2]$ l^{-1}). To assess the survival rate at 70 dph, fish were counted in each batch before the sex-ratio analysis.

To avoid mortality caused by cannibalistic behavior during the experiments, putative cannibals were removed from each aquarium as soon as this phenomenon was observed. A fish was considered as a cannibal when it reached a size more than 3 fold larger than the other individuals and can consequently hurt and/or consumed them. Cannibal removing was performed every morning before feeding.

2.3. Histological development of the gonad

At each sampling date (10, 15, 20, 25, 35, 45, 55 and 70 dph), 10 fish were randomly sampled and euthanized by an overdose (200 mg l^{-1}) of benzocaïne (Sigma-Aldrich). Fish were individually fixed in 10% buffered formalin (1:5 v/v) for at least 96 h. Heads and tails were cut and trunks incubated in propanol (Tissue-Tek VIP, Sakura, USA). Tissues were then embedded into paraffin, sectioned at a thickness of 5 μm with a rotary microtome (Reichert–Jung 2035, Leica) and stained with hematoxylin and eosin for histological observation.