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Sex determination and temperature-induced sex differentiation in three natural populations of Nile tilapia (*Oreochromis niloticus*) adapted to extreme temperature conditions

Etienne Bezault^{a,b,*}, Frédéric Clota^a, Martial Derivaz^a,
Bernard Chevassus^b, Jean-François Baroiller^a

^a CIRAD, Aquaculture & Aquatic Resource Management Unit, G.A.M.E.T., Avenue Agropolis-TA 30/01, F-34.398 Montpellier cedex 5, France

^b INRA, Laboratory of Fish Genetics, Domaine de Vilvert, F-78.352 Jouy-en-Josas, France

Abstract

As a species of major interest for aquaculture, the sex determination system (SDS) of Nile tilapia, *Oreochromis niloticus*, has been widely investigated. In this species, sex determination is considered to be governed by the interactions between a complex system of genetic sex determination factors (GSD) and the influence of temperature (TSD) during a critical period. Previous studies were exclusively carried out on domestic stocks with the genetic and maintenance limitations associated. Given the wide distribution and adaptation potential of the Nile tilapia, we investigated under controlled conditions the sex determination system of natural populations adapted to three extreme thermal regimes: stable extreme environments in Ethiopia, either cold temperatures in a highland lake (Lake Koka), or warm temperatures in hydrothermal springs (Lake Metahara), and an environment with large seasonal variations in Ghana (Kpandu, Lake Volta). The sex ratio analysis was conducted on progenies reared under constant basal (27 °C) or high (36 °C) temperatures during the 30 days following yolk-sac resorption. Sex ratios of the progenies reared at standard temperature suggest that the three populations share a similar complex GSD system based on a predominant male heterogametic factor with additional influences of polymorphism at this locus and/or action of minor factors. The three populations presented a clear thermosensitivity of sex differentiation, with large variations in the intensity of response depending on the parents. This confirms the presence of genotype-environment interactions in TSD of Nile tilapia. Furthermore the existence of naturally sex-reversed individuals is strongly suggested in two populations (*Kpandu* and *Koka*). However, it was not possible here to infer if the sex-inversion resulted from minor genetic factors and/or environmental influences. The present study demonstrated for the first time the conservation of a complex SDS combining polymorphic GSD and TSD components in natural populations of Nile tilapia. We discuss the evolutionary implications of our findings and highlight the importance of field investigations of sex determination. © 2007 Elsevier B.V. All rights reserved.

Keywords: *Oreochromis niloticus*; Sex determination; GSD; TSD; Natural populations; Sex-reversal

* Corresponding author. Department of Aquatic Ecology & Evolution, Institute of Zoology, University of Bern & Centre of Ecology, Evolution and Biogeochemistry, EAWAG, Seestrasse 79, CH-6047 Kastanienbaum, Switzerland. Tel.: +41 41 349 21 69; fax: +41 41 349 21 68.

E-mail addresses: etienne.bezault@eawag.ch, etienne.bezault@yahoo.fr (E. Bezault).

1. Introduction

Fish species are characterized by the diversity of their morphological, ecological and behavioural traits, and also of their sex determination systems (SDS). Morphologically differentiated sex chromosomes have been

reported in a minority of species (Traut and Winking, 2001). Furthermore, in most species sex-linked markers have also generally not been detected. Therefore, most of the present knowledge on sex determination system in fish has been obtained through chromosomal manipulation (gynogenesis, androgenesis, triploidization) or hormonal sex-reversal associated with progeny testing.

Various systems of sex determination have been observed in gonochoristic fishes (Baroiller et al., 1999; Devlin and Nagahama, 2002), spanning from a genetic sex determination system (GSD) with a simple genetic mono-factorial system, XX/XY or ZZ/ZW, to different polyfactorial systems or environmental sex determination (ESD). Temperature is the main environmental factor influencing the sex ratio in fish (*i.e.* temperature-dependent sex determination — TSD), as firstly demonstrated in the Atlantic silverside, *Menidia menidia* (Conover and Kynard, 1981) and later documented in a growing number of species from different families (Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002). Furthermore an important diversity of sex determination systems has been observed even between closely related taxa (*i.e.* intra-generic, inter-specific and even intra-specific) (Chevassus, 1998).

However, most studies on sex determination systems have been conducted on domestic stocks used for aquaculture purpose. Such *ex situ* stocks may have been genetically affected by various (fortuitous or not) historical events. First, bottlenecks, inbreeding and/or selection events could decrease the genetic diversity in some introduced and limited stocks by revealing lethal recessive genes or over-representing rare alleles (Shirak et al., 2002). Second, the generally high propensity of fishes for hybridization, combined to (1) the wide use of hybrids and synthetic strains in aquaculture (Bartley et al., 2001) and/or (2) the difficulty of accurate identification of closely related species in some groups, might have led to introgressions in some aquaculture or laboratory strains. The use of well characterized experimental stocks may avoid or limit impact of such genetic phenomena (Mylonas et al., 2005). However, the maintenance of broodstocks through several generations under controlled (artificial) conditions, sometimes far from those encountered by their wild population relatives, might affect the environmental component of the sex determination system, especially if these *ex situ* conditions inhibit or exacerbate expression of ESD (Lagomarsino and Conover, 1993).

The Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), is probably the best documented model species exhibiting a complex system of sex determination (Baroiller et al., 1995b; Baroiller and D'Cotta, 2001),

combining both genetic and environmental factors. The GSD system is based on a predominant mono-factorial genotypic system with male heterogamety (XX/XY) (Jalabert et al., 1974; Penman et al., 1987; Scott et al., 1989; Mair et al., 1991) and the influence of minor genetic factor(s) independent of (autosomal) and/or epistatic to the major sex determinant (Lester et al., 1989; Mair et al., 1991; Wohlfarth and Wedekind, 1991; Baroiller et al., 1996; Abucay et al., 1999; Baroiller and D'Cotta, 2001; Ezaz et al., 2004). The ESD component is based on the influence of temperature on sex differentiation with a functional masculinization of the female genotypes at high temperature (over 32–34 °C) (Baroiller et al., 1995b; Baras et al., 2001; Kwon et al., 2002; Tessema et al., 2006). In addition, possible genotype-environment interactions have been suggested (Baroiller et al., 1995b; Baroiller and Clota, 1998; Abucay et al., 1999; Baroiller and D'Cotta, 2001; Tessema et al., 2006).

The aforementioned studies were mainly based upon aquaculture domestic strains, such as *Bouaké* (Baroiller et al., 1995a,b; Baroiller and Clota, 1998) and *Manzala* (Mair et al., 1991; Wohlfarth and Wedekind, 1991; Baras et al., 2001), or even inter-specific crosses (Jalabert et al., 1971; Wohlfarth and Wedekind, 1991; Desprez et al., 2006), which could be widely affected by minor genetic and/or environmental factors as mentioned above. More recently, comparative studies were conducted on different domestic stocks, from Egypt, Ghana and Kenya (Abucay et al., 1999; Tessema et al., 2006), in order to investigate both genetic and environmental components of sex determination. Nevertheless, besides being propagated throughout numerous generations in *ex situ* environments, all these stocks originated from populations adapted to relatively similar climatic conditions, whereas the natural African distribution of Nile tilapia corresponds to a wide range of habitats with extreme thermal conditions (Philippart and Ruwet, 1982; Trewavas, 1983). In fact, Nile tilapia colonized environments varying from habitats with strong seasonal thermal variations, *e.g.* in West Africa, with alternation between hot (28–34 °C) and cold (22–26 °C) seasons (Talling, 2001), to altitude lakes with constant cold temperature (17–24 °C) (Admassu and Casselman, 2000), and hydrothermal hot springs (≥ 40 °C) (Trewavas, 1983). On the basis of the experimental results on TSD obtained under controlled conditions (Baroiller et al., 1995b; Baras et al., 2001; Kwon et al., 2002; Tessema et al., 2006), we hypothesized that such extreme natural thermal environments may differentially affect the system of sex determination in this species.

Table 1
Wild populations studied with geographic origin, drainage, GPS location, environmental conditions and number of breeders tested

Populations	Code	Drainage	Lake	Station	Lat.	Long.	Alt.	Thermal conditions	# Breeders	
									M	F
<i>O. n. cancellatus</i>										
Koka	Kk	Awash	L. Koka	Koka	08°24' N	39°01' E	1590 m	Cold	5	11
<i>O. n. filoa</i>										
Metahara	Me	Awash	L. Metahara	Abadir	08°51' N	39°50' E	955 m	Hot	4	1
<i>O. n. niloticus</i>										
Kpandu	Kp	Volta	L. Volta	Kpandu	06°48' N	00°18' E	85 m	Variable	7	10
Total									16	22

Therefore, the aim of the present study was to investigate, under controlled conditions, the sex determination system of three wild populations originating from contrasting thermal environments: constant low temperature (Lake Koka, Ethiopia), constant high temperature (Lake Metahara, Ethiopia) and seasonally variable temperatures (Lake Volta, Ghana). This was conducted by (1) searching for the existence of “naturally” sex-reversed individuals through progeny testing, and (2) comparing the relative importance of the genetic and environmental components in the sex determination system of this species.

2. Materials and methods

2.1. The study species

The main characteristics of the three natural populations and their environments are the following (see also details in Table 1):

- The cold-temperature population was sampled in Lake Koka (Kk), a reservoir of the Awash River in the Ethiopian Highlands. The thermal regime of Lake Koka is similar to Lake Awassa, where temperatures vary between 17 °C and 26 °C throughout the year (Admassu and Casselman, 2000). During the sampling period (May 2002), the surface water temperature varied between 21 °C and 26 °C during the day.
- The population of Lake Metahara (Me) was chosen because it is adapted to high temperature conditions. This isolated lake (associated to the Awash River system) is directly supplied by hot waters flowing (10 L/s) from hydrothermal hot springs at a temperature of 43 °C (Goërmer et al., 2005). Based on satellite imaging, superficial water temperature of the lake varied between 30.4 °C and 40 °C (Goërmer et al., 2005). Nile tilapias were collected a few meters from these hot springs, where temperatures varied between 32 °C and 39 °C (in May 2002).

C) For populations adapted to large seasonal temperature fluctuations, we focused on the Volta basin in Ghana. The selected population originated from Kpandu (Kp), a dendritic expansion on the eastern side of Lake Volta. At this site, temperature in open water may vary monthly between 27 °C to 32 °C along the year (Bezault, 2005) and it may warm up by several additional degrees in the shallow habitats where fry spend their critical period of sex differentiation.

Further molecular analysis revealed a clear genetic differentiation between these three populations of Nile tilapia ($F_{st}=0.378\pm 0.070$), including between the two from the Awash basin ($F_{st}=0.287$) (Bezault, 2005).

All the individuals were collected in the wild when sexually mature (about 50 to 120 g), during May 2002 for the Ethiopian populations and between April 2002 and March 2003 for Ghanaian one. They were then transferred to the experimental facilities of the GAMET (Montpellier, France), and progressively acclimated to a standard temperature of 27 °C \pm 1 °C in a thermo-regulated re-circulating system. Fish were individually tagged with passive integrated transponders (PIT-tags; MicroBE).

Within each stock, a semi-factorial crossing plan was performed using randomly chosen breeders in order to evaluate the parental influences while testing a rather large number of breeders. However, for the *Metahara* stock, due to high mortality (caused by a high parasitic load) only 4 males and 1 female could be studied. To overcome the lack of females from this stock, the *Metahara* males were crossed with females either (1) of known sexual genotype from the domestic *Bouaké* strain maintained in the GAMET (*i.e.* two independent XX females, not tested previously for their thermosensitivity; leading to “XM” progenies) or (2) from the genetically closest wild population, even though it is the most divergent population from an ecological point of view (*i.e.* *Koka*; leading to “KM” progenies). Then the pure *Metahara* progenies were labelled “ME” and in the study the term of “*Metahara* (Me)” was used for the total of the different crosses involving at least one breeder from *Metahara* (*i.e.* Me = ME + KM + XM).

2.2. Experimental protocol

Progenies were obtained either by natural or *in vitro* fertilization methods.

For natural breeding, three females were stocked in a 240 L aquarium together with a single male. Fish reproduced freely but were constantly surveyed in order to check that a single female spawned at a time and collected its fertilized eggs (no multiple spawning was observed during the entire study). Soon after spawning, the female was gently isolated from the other breeders in the aquarium. After three days of mouth-brooding, the female was cautiously captured and the eggs were collected from her mouth. Fry were then transferred into a McDonald jar for incubation at $27\text{ }^{\circ}\text{C}\pm 1\text{ }^{\circ}\text{C}$, until the free swimming stage.

For the *in vitro* fertilization, female breeders were selected based on their reproductive behaviour and on the development of their urogenital papilla. Selected females were cautiously stripped under anesthesia (with 2-Phenoxyethanol, at the dosage of 1.5 mL for 10 L of water). Fertilizations were performed according to Chourrout and Itskovitch (1983). Eggs were then incubated in a McDonald jar, as described above.

At the free swimming stage, around 9–10 days post-fertilization (PF), progenies were randomly divided into two groups (limited to 180 larvae per group for the largest progenies), and transferred into 40 L tanks thermo-regulated at either $27\text{ }^{\circ}\text{C}$ (control) or $36\text{ }^{\circ}\text{C}$ (treatment). Thermal treatment extended over 30 days, in order to observe the optimal influence on sex differentiation (Baroiller et al., 1995b). Then the groups were transferred for the on-growing period into 80 L tanks at control temperature ($27\pm 1\text{ }^{\circ}\text{C}$) and were raised for 60 additional days until sexing.

Throughout the treatment and rearing periods, water temperature in the different tank systems was continuously surveyed with data loggers (TidbiT, Onset Computer Corporation; accuracy of $0.3\text{ }^{\circ}\text{C}$). Dissolved oxygen was also monitored periodically and maintained at saturation in both (control and treated) conditions. Fish were counted at the beginning of the experiment, at the end of the treatment, and at sexing, in order to determine mortality during and after treatment.

At 90 days PF, sex ratios were determined by gonadal squashes, on a random sample of 100 individuals per group, or the entire group if less than 100 fish survived at the time of sexing. Fish were sacrificed by administration of a lethal dosage of anaesthetic (2-Phenoxyethanol at a concentration of 1 mL per liter of water).

The experiments lasted from December 2003 to October 2004.

2.3. Statistical analysis

For each progeny, the sex ratio (SR) was expressed as the proportion (%) of males over the total number of sexed individuals. The effect of temperature on sex ratio was expressed in two ways: (1) the difference between the sex ratios of the treated ($36\text{ }^{\circ}\text{C}$) and the control ($27\text{ }^{\circ}\text{C}$) groups for a given progeny ($D_{\text{SR}} = \text{SR}_{36} - \text{SR}_{27}$); (2) the masculinization efficiency of the thermal treatment (EM_{T}), estimated by the increase of male

proportion in treated conditions as a proportion of females in the control group:

$$\text{EM}_{\text{T}} = (\text{SR}_{36} - \text{SR}_{27}) / (100 - \text{SR}_{27})$$

The sex ratio of each group (both control and treated) was compared with the expected balanced (1:1) sex ratio (and in unbalanced cases, to skewed (3:1) sex ratio — *data not shown*). The effect of the treatment on the progeny was tested by comparison between sex ratio of the treated and the control groups using a 2×2 contingency Chi-square test. Chi-square tests were also used to test for the effect of thermal treatment (during and after treatment) on the mortality rate.

Within each of the three populations under study, the overall effect of temperature on sex ratio and mortality was tested with a Wilcoxon rank test.

At the population level, the distribution (*i.e.* mean and variance) of the sex ratio was investigated as follows:

- To test if the mean sex ratio of each population at control temperature ($27\text{ }^{\circ}\text{C}$) differed significantly from 50% of males (*i.e.* balanced SR), the confidence interval (CI) of the mean sex ratio of the population was calculated. If the value 50% was not within the CI, we concluded a significant deviation was present.
- To test if the inter-familial variance in the sex ratio, within each population, was significantly higher than expected under a binomial distribution, the CI of this observed variance was compared with the theoretical value (V_{th}) under the hypothesis that the inter-familial variance reflected only sampling fluctuations of a frequency f obtained over samples of 100 sexed individuals (*i.e.* $V_{\text{th}} = f \times (1-f) / 100$). If the lower boundary of the calculated CI was higher than V_{th} , we concluded that there was a significant variation in the sex ratio between families.

Furthermore, to analyse this inter-family variation over all the progenies, the distribution of observed sex ratios (divided in five classes reflecting this distribution: 0 to 40%; 40 to 50%; 50 to 55%; 55 to 60% and 60 to 100%) was compared to a normal distribution with a mean of 50% and a theoretical binomial variance by a Chi-square test with 4 degrees of freedom (df).

Between-population comparisons of mean sex ratio (for both control and treated samples), mortality (during and after treatment), differences in SR (D_{SR}) and masculinization efficiency (EM_{T}) were performed using 2×2 Wilcoxon rank tests.

Pair-wise comparisons of inter-familial variances of sex ratio (between populations and between temperatures) were performed using Snedecor F tests.

Correlations between each of the two estimators of thermosensitivity (D_{SR} and EM_{T}) and difference of mortality rates (during and after treatment) were tested by non-parametric Spearman correlation analysis.

3. Results

3.1. Experimental treatment

A total of 57 progenies were analyzed: 21 from *Kpandu*, 18 from *Koka* and 18 from *Metahara* (including 4 pure- and 14 cross-breed progenies). These crosses involved a total of 38 wild breeders plus 2 XX females from the *Bouaké* strain (Tables 1 and 2).

The mean observed temperatures in the control and treatment groups did not differ from the target values (26.9 ± 1.58 °C and 36.0 ± 0.45 °C respectively).

3.2. Mortality

The mortality rates over 30 days of thermal exposure at 27 °C and 36 °C were, respectively, 10% and 17%, versus 12% and 4% during the following 60 days of growing (Table 2).

Within populations (Table 2), no significant differences related to the thermal condition during the period of sex differentiation were found in *Kpandu* (neither during this period nor after), whereas differences appeared in *Koka* and *Metahara*: mortalities were significantly higher in treated groups during treatment and conversely higher in control groups during the growing period. In the *Metahara* population, differences were observed between the different crossing types. There were no temperature-dependent differences between the mortalities of pure progenies (ME) or among crosses with breeders of the *Bouaké* strain (XM). Conversely, the progenies involving *Koka* females (KM), which were the majority of the crosses involving *Metahara* breeders (11 out of 18), presented a greater mortality at a temperature of 36 °C than at 27 °C, during the thermal treatment. Globally all these

results suggest a higher sensitivity of the Lake Koka population to high temperatures rather than the two other studied populations.

Inter-population comparisons of mortality during and after the differentiation period were carried out for each treatment (Table 3). At 27 °C, progenies from *Kpandu* stock showed a greater mortality than those of the other two populations during the gonadal differentiation period, whereas no significant differences could be seen during the growing period. At high temperature (36 °C), no significant differences were present during the gonadal differentiation period; the only significant differences were observed between *Kpandu* and *Metahara* progenies during the growing period (with a lower mortality in *Metahara*).

3.3. Mean sex ratios

At control temperature, although two populations presented almost balanced mean sex ratios (*i.e.* 50.2% males in *Kpandu*, 52.9% in *Metahara*), the third population exhibited a significantly skewed sex ratio in favour of males (55.8% for *Koka*) (Table 2).

When exposed to high temperature, all three populations produced globally relatively similar skewed sex ratios. The average proportions of males were 78.7% in *Kpandu*, 80.6% in *Metahara* and 77.0% in *Koka*. All are highly significantly different from balanced sex ratios, with no significant differences between populations (Table 3).

Comparisons between sex ratios obtained at 27 °C and 36 °C showed a very highly significant effect of the temperature for the three populations (Table 2) but no significant differences in the mean thermosensitivity (*i.e.*

Table 2

Distribution of sex ratio (mean, variance, minimum and maximum), mortality rates (mean & standard deviation), during and after treatment, in function of population and temperature treatment, in regard to the number of family tested (Num. fam.); the distribution of sex ratio was compared to theoretical distribution: for the mean sex ratio at standard temperature to balanced (1:1) sex ratio and for its variance for both temperatures to a normal distribution (mean=0.5 and binomial variance); effects of temperature treatment on sex ratio and mortality rates were tested by Wilcoxon rank test within each population

Pop.	T°	Num. fam.	Sex ratio							Mortality during treat.			Mortality post-treat.		
			Mean		Variance	Min.	Max.	P	Mean	St. dev.	P	Mean	St. dev.	P	
Total	27	57	52.8	NS	137.6	**	4.9	77.7	–	0.10	0.152	–	0.12	0.167	–
	36	57	78.8	–	201.2	**	43.1	100	–	0.17	0.152	–	0.04	0.052	–
Kp	27	21	50.2	NS	256.5	**	4.9	77.7	***	0.22	0.208	NS	0.08	0.079	NS
	36	21	78.7	–	223.5	**	53	100	–	0.15	0.150	–	0.05	0.044	–
Kk	27	18	55.8	**	70.7	**	45.5	72	***	0.05	0.056	**	0.17	0.243	*
	36	18	77	–	284.8	**	43.1	100	–	0.15	0.154	–	0.05	0.074	–
Me	27	18	52.9	NS	62.9	**	38.5	68	***	0.04	0.047	***	0.12	0.133	**
	36	18	80.6	–	110.6	**	52.1	94	–	0.22	0.150	–	0.02	0.018	–
ME	27	4	54.1	–	32.2	–	49	61	***	0.09	0.054	NS	0.10	0.089	NS
	36	4	83.6	–	38.1	–	75	89	–	0.20	0.226	–	0.04	0.026	–
XM	27	3	51.3	–	50.3	–	45	59	NS	0.04	0.059	NS	0.05	0.061	NS
	36	3	72.3	–	60.3	–	66	81	–	0.07	0.037	–	0.01	0.000	–
KM	27	11	52.9	–	86.1	–	38.5	68	***	0.02	0.02	***	0.15	0.157	**
	36	11	81.7	–	139.9	–	52.1	94	–	0.26	0.116	–	0.01	0.013	–

Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, NS for $P > 0.05$ and “–” not tested.

with both estimators, D_{SR} and EM_T) between populations. Furthermore no correlation was observed between differences in sex ratios and mortality according to the temperature conditions (Table 4).

3.4. Sex ratio variation

Overall for the studied progenies ($n=57$), the between family variability of the observed sex ratio obtained at control temperature was very significantly higher ($P<0.001$) than a theoretical distribution (mean 50% and binomial variance), particularly with an over-representation of progenies with a sex ratio higher than 60% males (*i.e.* 19.3% in the observed distribution versus 2.3% under theoretical distribution) (Table 5).

This high variability of sex ratios was observed at both control and treated temperatures within each of the three populations (Table 2). At 27 °C, *Kpandu* presented a larger variability than the two other populations. At 36 °C, the *Metahara* population had a significantly lower variability while the variability of the *Koka* population increased dramatically to reach a level similar to *Kpandu*, which presented the same variability at both temperatures (Table 6).

Given this significant variation in the sex ratios at both control and masculinizing temperatures, more precise intra-population investigations were undertaken.

Within the *Koka* population (Table 7a) most of the progenies showed balanced sex ratios at the control temperature (14 out of 18 progenies, 77.8%). The four unbalanced sex ratios appeared to be in agreement with a (3:1) male-biased sex ratio. Three of the four cases (Kk-05; Kk-07; Kk-08) were sired by the same male Kk-M39 and their respective maternal half-sib families did not present such skewed sex ratio, this

Table 3
Inter-population comparisons of a) sex ratio and mortality rates, b) during and c) after treatment, at both temperature treatments (27 °C and 36 °C), by Wilcoxon rank test; both probability and degree of significance are reported

	Control (27 °C)			Treatment (36 °C)		
	Kp	Kk	Me	Kp	Kk	Me
<i>a) Sex ratio</i>						
Kp		0.4549	0.9662		0.7036	0.8546
Kk	NS		0.4286	NS		0.5906
Me	NS	NS		NS	NS	
<i>b) Mortality rate — during treatment</i>						
Kp		0.012	0.0057		0.9515	0.1762
Kk	*		0.4056	NS		0.1328
Me	**	NS		NS	NS	
<i>c) Mortality rate — post-treatment</i>						
Kp		0.3699	0.5133		0.3595	0.0035
Kk	NS		0.8001	NS		0.2592
Me	NS	NS		**	NS	

Significance: * $P<0.05$; ** $P<0.01$; *** $P<0.001$, NS for $P>0.05$.

Table 4

Correlations between thermosensitivity estimators (difference of sex ratio between temperatures, $D_{SR}=SR_{36}-SR_{27}$; masculinization efficiency, EM_T) and difference of mortality rates between temperature treatment, during and after the thermal treatment, performed by non-parametric Spearman method

	DSR	EMT	DMort(Treat.)	DMort(Post-T.)
DSR		0.4415	0.1004	0.1276
EMT	***		0.1432	0.0216
DMort(Treat.)	NS	NS		0.1095
DMort(Post-T.)	NS	NS	NS	

Significance: * $P<0.05$; ** $P<0.01$; *** $P<0.001$, NS for $P>0.05$.

observation rejecting the hypothesis of spontaneous XY females for F04, F06 and F20. The fourth male-skewed progeny (Kk-14) was produced by the female Kk-F10, which also produced another male-skewed progeny in an inter-population cross (KM-17), sired by a *Metahara* male (Me-M15), suggesting a possible XY genotype for this female. At high temperature, male-skewed sex ratios were observed in 16 out of 18 progenies (88.9%), but significant effects of temperature on sex ratio were detected only in 11 progenies (61.1%).

Within the *Metahara* population (Table 7b) most of the progenies showed balanced sex ratios at the control temperature (13 out of 18 progenies, 72.2%). Within the four pure-breeds (ME), all presented balanced and male-biased sex ratios respectively at control and high temperature and significant differences in the sex ratios according to the treatment; the only exception was Me-03 which showed a slight excess of males at the control temperature. The cross-breeds with females from the *Bouaké* strain (XM) presented the same expected pattern (except for XM-02, for which the sex ratio difference was not significant). Likewise, the cross-breeds with females from the *Koka* population (KM) mainly showed the same expected patterns. However, four families presented significant deviations from a balanced sex ratio in control groups: three were slightly skewed in favour of males (KM-01, KM-03 and KM-17) and one in favour of females (KM-10). Considering the respective half-sib families, the simple hypothesis of

Table 5

Comparison of the distribution of observed sex ratio over all the progenies, at standard temperature (27 °C), to an expected normal distribution (with a mean of 0.5 and a binomial variance), by Chi-square test; observations of the sex ratio distribution were divided into 5 classes (*i.e.* 4 *df*)

Sex-ratio classes	# observed	# expected	Chi ² value
0–39.9%	3	1.3	2.22
40–49.9%	16	27.2	4.61
50–54.9%	16	19.4	0.6
55–59.9%	11	7.8	1.31
60–100%	11	1.3	72.38
Total	57	57	81.12***

Significance: * $P<0.05$; ** $P<0.01$; *** $P<0.001$, NS for $P>0.05$.

Table 6
Pair-wise comparison of inter-familial variance of sex ratios using Snedecor *F* test

	Kpandu	Koka	Metahara	Kpandu corr
Var 27 °C	256	71	63	66
Var 36 °C	223	285	111	197
Kpandu	NS	**	**	**
Koka	NS	**	NS	NS
Metahara	NS	*	NS	NS
Kpandu corr	NS	NS	NS	*

Diagonal: comparison between 27 °C and 36 °C for each population; above diagonal: between population comparisons at 27 °C; below diagonal: between population comparisons at 36 °C. Kpandu corr = after three progenies of a spontaneous XX male were removed. Significance: **P*<0.05; ***P*<0.01; ****P*<0.001, NS for *P*>0.05.

spontaneous YY males in *Metahara* population can be rejected, however the hypothesis of spontaneous XY females in *Koka* population can be accepted for the female Kk-F10 (as previously mentioned for KM-17), but it has to be rejected for the three other groups. At high temperature, male-skewed sex ratios were observed in 17 out of 18 progenies (94.4%), but significant effects of temperature on sex ratio were detected only in 15 progenies (83.3%).

Within the *Kpandu* population (Table 7c) a high majority of the progenies showed balanced sex ratios at the control temperature (17 out of 21 progenies, 81%). However four progenies presented deviations from the expected balanced sex ratios. Two unrelated families (Kp-01 and Kp-18) showed male-biased sex ratios; however their respective paternal half-sib progenies did not show any bias, indicating a putative female effect (Kp-F02 and Kp-F50, respectively) or an interaction between breeders (Kp-F02 with Kp-M09 and Kp-

Table 7a

Crossing plan of the studied progenies from the *Koka* populations; for each cross are given the sex ratio (SR) at control (27 °C) and high (36 °C) temperatures, Chi-square tests were used to compare (1) independence of each observed sex ratio from the expected balance (1:1) and (2) homogeneity of the sex ratio between treatments within each progeny (*D*_{SR})

Females	Males	Kk-M01		Kk-M03		Kk-M05		Kk-M09		Kk-M39			
		27 °C	36 °C	27 °C	36 °C	27 °C	36 °C	27 °C	36 °C	27 °C	36 °C		
Kk-F04	Fam. SR <i>D</i> _{SR}					55	NS 84	***		72	*** 98	***	

Kk-F06	Fam. SR <i>D</i> _{SR}								49	NS 74	*	67	*** 100
										*		***	
Kk-F08	Fam. SR <i>D</i> _{SR}	55	NS 62	*		57	NS 43	NS					
			NS				NS						
Kk-F10	Fam. SR <i>D</i> _{SR}					70	*** 80	***					
							NS						
Kk-F12	Fam. SR <i>D</i> _{SR}	55	NS 73	***		55	NS 58	NS					
			**				NS						
Kk-F16	Fam. SR <i>D</i> _{SR}					49	NS 64	*					
							NS						
Kk-F20	Fam. SR <i>D</i> _{SR}					58	NS 60	*			68	** 99	***
							NS					***	
Kk-F22	Fam. SR <i>D</i> _{SR}								47	NS 60	*		
										NS			
Kk-F24	Fam. SR <i>D</i> _{SR}	50	NS 92	***		49	NS 70	***					
			***				**						
Kk-F26	Fam. SR <i>D</i> _{SR}					46	NS 84	***			54	NS 97	***
							***					***	
Kk-F28	Fam. SR <i>D</i> _{SR}								47	NS 87	***		

Significance: **P*<0.05; ***P*<0.01; ****P*<0.001, NS for *P*>0.05.

Table 7b

Crossing plan of the studied progenies from the *Metahara* populations; for each cross are given the sex ratio (SR) at control (27 °C) and high (36 °C) temperatures, Chi-square tests were used to compare (1) independence of each observed sex ratio from the expected balance (1:1) and (2) homogeneity of the sex ratio between treatments within each progeny (D_{SR})

Females	Males	Me-M01		Me-M03		Me-M13		Me-M15	
		27 °C	36 °C	27 °C	36 °C	27 °C	36 °C	27 °C	36 °C
Me-F02	Fam. SR D_{SR}	49	Me-05 NS 89 *** ***	61	Me-03 * 75 *** *	50	Me-04 NS 87 *** ***	57	Me-02 NS 83 *** ***
Bk -F01	Fam. SR D_{SR}					45	XM-01 NS 70 *** ***		
Bk -F02	Fam. SR D_{SR}			50	XM-03 NS 81 *** ***			59	XM-02 NS 66 ** NS
Kk -F04	Fam. SR D_{SR}			65	KM-01 ** 92 *** ***				
Kk -F06	Fam. SR D_{SR}	51	KM-09 NS 91 *** ***						
Kk -F10	Fam. SR D_{SR}							68	KM-17 *** 85 *** **
Kk -F12	Fam. SR D_{SR}	52	KM-06 NS 94 *** ***			65	KM-03 ** 79 *** NS		
Kk -F16	Fam. SR D_{SR}	38	KM-10 * 52 NS NS						
Kk -F20	Fam. SR D_{SR}							43	KM-16 NS 71 *** ***
Kk -F22	Fam. SR D_{SR}							47	KM-14 NS 87 *** **
Kk -F24	Fam. SR D_{SR}					53	KM-02 NS 82 *** ***	49	KM-07 NS 84 *** ***
Kk -F26	Fam. SR D_{SR}			51	KM-15 NS 82 ***				

Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, NS for $P > 0.05$.

F50 with Kp-M19, respectively). On the other hand, two other progenies (Kp-13 and Kp-22), sired by the same male parent (Kp-M23), showed strong excesses of females at the control temperature. However, their maternal half-sib progenies presented balanced sex ratios; this tends to suggest a paternal effect. Furthermore the cross between the male Kp-M23, expected to produce an excess of females, with the female Kp-F50, expected to produce male-biased progenies (3:1), showed a balanced sex ratio. Such opposite deviations in sex ratio and the complementation of both parental effects seem to be in agreement with the hypothesis of sex-reversed individuals for these two breeders. Under this hypothesis, Kp-M23 would be a natural pseudo-male (XX $\Delta\sigma$) and Kp-F50 a natural pseudo-female (XY $\Delta\varphi$). Kp-F02 could be another natural pseudo-

female but no maternal half-sib progeny was available to confirm this hypothesis. At high temperature, male-skewed sex ratios were observed in 19 out of 21 progenies (90.5%), but significant effects of temperature on sex ratio were detected only in 15 progenies (71.4%).

Removing the 3 progenies of the pseudo-male XX (Kp-M23) in the *Kpandu* population drastically changed some conclusions of the former analysis of between-population differences. First, the overall sex ratio in this population became significantly skewed in favour of males (54.9%), as in the *Koka* population, and no more significant differences of mean sex ratios were observed between the three populations. Secondly, comparisons of between family variability of sex ratios (Table 6) gave rather different results: at 27 °C, the high

Table 7c

Crossing plan of the studied progenies from the *Kpandu* populations; for each cross are given the sex ratio (SR) at control (27 °C) and high (36 °C) temperatures, Chi-square tests were used to compare (1) independence of each observed sex ratio from the expected balance (1:1) and (2) homogeneity of the sex ratio between treatments within each progeny (D_{SR})

Females	Males	Kp-M09		Kp-M15		Kp-M17		Kp-M19		Kp-M21		Kp-M23		Kp-M27	
		27 °C	36 °C	27 °C	36 °C	27 °C	36 °C	27 °C	36 °C	27 °C	36 °C	27 °C	36 °C	27 °C	36 °C
Kp-F02	Fam. SR D_{SR}	79	Kp-01 91 NS												
Kp-F04	Fam. SR D_{SR}	54	Kp-02 66 NS												
Kp-F32	Fam. SR D_{SR}					46	Kp-20 NS 88 ***	56	Kp-17 NS 89 ***			5	Kp-13 *** 71 ***		
Kp-F34	Fam. SR D_{SR}		Kp-12	60	NS 53 NS									49	Kp-16 NS 71 **
Kp-F40	Fam. SR D_{SR}	47	Kp-04 NS 69 **			50	Kp-05 NS 55 NS	49	Kp-06 NS 91 ***						
Kp-F46	Fam. SR D_{SR}					50	Kp-19 NS 69 *			58	Kp-15 NS 71 NS				
Kp-F48	Fam. SR D_{SR}		Kp-07	50	NS 68 *			58	Kp-08 NS 95 ***					54	Kp-14 NS 91 ***
Kp-F50	Fam. SR D_{SR}							69	Kp-18 *** 100 ***			51	Kp-21 NS 100 ***		
Kp-F52	Fam. SR D_{SR}		Kp-11	47	NS 65 *							11	Kp-22 *** 99 ***		
Kp-F54	Fam. SR D_{SR}									58	Kp-10 NS 67 NS			54	Kp-09 NS 89 ***

Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, NS for $P > 0.05$.

observed variance in the *Kpandu* population decreased significantly to a value similar to the two other populations. On the other hand, variance remained high at 36 °C. A significant increase of variability was then observed both in the *Koka* and *Kpandu* populations but not in the *Metahara* population.

4. Discussion

Although two previous studies aimed to compare the sex determination system of Nile tilapia among different experimental stocks (Abucay et al., 1999; Tessema et al., 2006), the present study appears to be the first one based on wild breeders originating from well differentiated thermal habitats.

4.1. Genetic sex determination

At the control temperature, the distribution of sex ratios over all the tested progenies showed a more important variability than expected under the binomial distribution of balanced mean sex ratios. Within populations, the progenies of all three wild populations exhibited a very high variability of sex ratios when exposed to basal temperature (27 °C): 4.9–77.7% for *Kpandu*, 45.5–72.0% for *Koka* and 38.5–68.0% for *Metahara*. 19 to 28% of families presented significantly skewed sex ratios with a large majority of these families (9 out of 12) being skewed in favour of males, depending on the populations. Unlike previous studies proposing that sex ratios of tilapias exhibited an almost normal distribution within populations (Shelton et al., 1983; Mair et al., 1991), the present results support the conclusion of a more important variation of sex ratios within natural populations regardless of the temperature treatment.

The sex ratios in crosses between populations did not present any significant discrepancy in comparison to the pure-breeds, which is in accordance with the view that all of the natural populations under study share the same predominant monofactorial sex determination system. Nevertheless, the considerable variability between the sex ratios of the different progenies suggests that the mechanism of genetic sex determination is more complex, either due to polymorphism at the major sex determinant (multi-allelic) and/or the action of minor genetic factor(s), either autosomal or linked to the major sex determinant, as suggested in other studies highlighting such predominant parental influences (Baroiller et al., 1996; Baras et al., 2001). Furthermore, at the individual level, the present results show the existence of parental effects with both paternal (e.g. Kk-M39) and

maternal effects (e.g. Kk-F10) or even breeding pair interaction (e.g. family KM-03 descendant from Me-M13 and Kk-F12) on the sex ratios, in agreement with previous findings (Baroiller, 1996; Baroiller and Clota, 1998).

A more precise focus on the highest skewed sex ratios, led to hypothesize peculiar sex determinant genotype and/or association between sexual genotype and phenotype for several of the breeders under study. In the *Koka* population, the existence of a spontaneous XY female can be hypothesized (Kk-F10 $\Delta^{\text{♀}}$ XY) but this simple model does not explain the skewed sex ratios observed in some of the progenies from other breeders (male Kk-M39, females Kk-F04, F06, F12, F16 and F20), for which strong influences of secondary genetic factors should be assumed. The results obtained with *Kpandu* breeders seem to be clearly in favour of sex-reversed individuals for both Kp-M23 ($\Delta^{\text{♂}}$ XX) and Kp-F50 ($\Delta^{\text{♀}}$ XY) and possibly for female Kp-F02 ($\Delta^{\text{♀}}$ XY). The occurrence of such spontaneous sex-reversed individual was pointed out once by Scott et al. (1989): these authors have identified through gynogenesis within a non-treated broodstock one phenotypic female assumed to be a genotypic male ($\Delta^{\text{♀}}$ XY). Such hypothesis was confirmed by the identification of “super-male” genotypes (♂ YY) within her progeny when mated with a classic male (♂ XY). Mair et al. (1991) also observed 2 families out of 59 with male-skewed sex ratios possibly resulting from spontaneous XY females. The present results confirm for the first time in the wild the existence of natural sex-reversed specimens of Nile tilapia at least at the level of major sex determinant(s).

4.2. Temperature-induced sex differentiation

The three studied populations present a widespread thermosensitivity: 91.2% of progenies exhibited an excess of males at high temperature and 71.9% a significant increase of this proportion in comparison with the control temperature. The hypothesis of differential mortality to explain bias of sex ratio can be rejected here as already demonstrated in previous studies using genetically mono-sex female populations of this species (Baroiller et al., 1995b; Baroiller et al., 1996; Abucay et al., 1999) or other tilapia species (Baroiller et al., 1995a; Desprez and Mélard, 1998b,a; Baroiller and D’Cotta, 2001). Another irrefutable demonstration of temperature-induced sex-inversion has been provided by progeny testing of temperature-treated males; some of them gave rise to almost 100% female progenies (Baroiller et al., 1995b). Therefore,

most if not all skewed sex ratios induced by high temperature treatments correspond to functional masculinization of genetic females.

However a significant proportion of crosses within each population did not show any significant increase of male proportions following thermal treatment (28.6% for *Kpandu*, 38.9% for *Koka* and 16.7% for *Metahara*) and within them a smaller part exhibited balanced sex ratios at high as well as at low temperature (9.5% for *Kpandu*, 11.1% for *Koka* and 5.6% for *Metahara*). Almost complete temperature-induced masculinization (proportions of males $\geq 90\%$) also appears to be in a minority (16.7% of families for *Metahara*, 27.8% and 33.3% for *Koka* and *Kpandu* respectively). Even with thermal conditions assumed to maximize temperature-induced sex differentiation, this leads to a relatively moderate average population sex ratio (from 77.0% to 80.6% of males), far from a total inversion at the population level.

The present results, showing large variation of thermosensitivity within populations, are in agreement with previous studies based upon aquaculture or experimental strains (Mair et al., 1991; Wohlfarth and Wedekind, 1991; Baroiller et al., 1995b; Abucay et al., 1999; Baras et al., 2001; Tessema et al., 2006). However, some differences in variability of thermosensitivity have been observed between the populations in the present study. The *Metahara* population, adapted to high temperature in the wild, presents the more homogeneous response to thermal treatment in terms of the sex ratio and, at the extreme opposite, a large increase of sex ratio variability at high temperature has been observed within the *Koka* population, adapted to constant low temperature. A similar but smaller increase in variability was observed in the *Kpandu* population, after removing the effect of the spontaneous XX male detected in this population. The lower variability observed in the *Metahara* population might result either from a better adaptation to high temperature and/or from a “heterosis effect”, since most progenies (14 out 18) resulted from crosses with females of other populations (*Bouaké* and *Koka*).

However, no relationship was established here between the sex ratio of progenies at control temperature (or the departure from a balanced sex ratio) and the propensity to be masculinized by high temperatures. Such inter-familial variation points to important parental effects and genotype-environment interactions as suggested by other studies (Baroiller et al., 1995b; Baroiller and Clota, 1998; Abucay et al., 1999; Tessema et al., 2006). Such parental effects have also been found in other thermosensitive species such as *M. menidia* (Conover and Heins, 1987) and *Odonthestes bonariensis* (Strüssmann et al., 1996).

Furthermore, other studies have proved that high temperatures can have both masculinizing and feminizing effects depending on the sex genotype (Abucay et al., 1999; Baroiller and D’Cotta, 2001; Kwon et al., 2002). In the present work, only significant increases in male proportions have been demonstrated (even in Kp18, hypothesized to be mainly composed of males (3:1), in which a total masculinization appears at high temperature). However the present results cannot reject the possibility of a potential feminization of some male genotypes by high temperature. Nevertheless, within the *Bouaké* strain, progeny testing of females originating from temperature-treated classic progenies did not reveal any sex-reversed XY females (Baroiller, unpublished data), whereas other works demonstrated the presence of XX males in temperature-treated progenies (Baroiller et al., 1995b). This suggests that putative feminizing effect of high temperatures is probably restricted to peculiar genotype (e.g. YY, rather than XY) and/or population/strain/species.

4.3. A complex model of sex determination and evolutionary implications

This study provides evidence of a complex polymorphic sex determination system combining both GSD and TSD in the three different natural populations of Nile tilapia adapted to extreme thermal regimes. These populations appear to be genetically well differentiated and not to have been introgressed or suffered experimental inbreeding (Bezault, 2005). To our knowledge, this is the first evidence that the natural populations of tilapia can also present a thermosensitivity of their gonadal sex differentiation. Additionally it appears also to be the first evidence of spontaneous phenotypic sex-reversed individuals (of both sexes) demonstrated at least in two different natural populations. However the cause of this sex-reversal could not be clearly attributed to a genetic effect of minor determinant(s) and/or to an influence of the temperature conditions. These results suggest a more important influence of the various minor factors, genetic as well as environmental, within the complex system of sex determination of this species, than previously reported. In fact, in all strains (or populations) of Nile tilapia studied to date, sex determination is seemingly governed by the following three categories of factors as listed by Bull (1983, 2005): (1) a predominant system of major genetic determinant (here: XX/XY); (2) an effect of minor sex determinant(s) or modifier factor(s) and (3) the influence of environmental parameters during the critical phase of sex differentiation (here: high water temperature with genotype-environment interactions).

At the population level, thermosensitivity as well as genetic factors (major and minor) provide a large variability of sex ratios. Nevertheless all or almost mono-sex progenies were not observed in the present study. This corroborates the hypothesis that truly mono-sex progenies induced by environmental factors are exceptional among gonochoristic fish species (Strüssmann et al., 1996; Baroiller et al., 1999; Baroiller and D'Cotta, 2001; Conover, 2005). The existence of sex determination mechanisms providing no straightforward or complete response to the variations of environmental conditions, such as water temperature, might be regarded as a consequence of environmental unpredictability, which generated no unidirectional selection pressure against a particular type of mechanism (Baras et al., 2001, 2002). From a functional point of view, such a complex mechanism might be regarded as a conservative way of resisting sudden environmental changes without compromising the chances of adapting again to environments encountered previously in case of cyclic changes (Baras, personal communication).

Our experiments were conducted in the very same way as other studies on thermolabile sex differentiation in Nile tilapia, *i.e.* 1) challenge under constant thermal conditions over 30 days at either basal temperature (27 °C), in order to observe sex ratio without environment influence (*i.e.* pure GSD); and 2) challenge at a high temperature (36 °C), which was demonstrated to be sufficient to widely masculinize progenies in other populations of this species (Baroiller et al., 1995b; Abucay et al., 1999; Baras et al., 2001; Tessema et al., 2006). However, it has been shown that the threshold of maximal thermal influence on sex differentiation may vary between species and strains (*i.e.* 34°–35 °C for *Oreochromis aureus*, 36 °C for *O. niloticus* from Bouaké and 37 °C for *O. niloticus* from Manzala) (Baroiller et al., 1995b; Baras et al., 2001; Baras et al., 2002). It is possible that such variations of thermal threshold of thermosensitivity may occur within populations, too.

In addition, such long term constant temperature regimes contrast with the highly fluctuating temperatures recorded in the shallow water habitat where tilapias fry shoals concentrate during their critical stage of sex differentiation (Baroiller et al., 1995b; Bezault, 2005), raising the question of an effective influence of temperature on sex ratios in these conditions. Nevertheless, Baras et al. (2000) have demonstrated that nycthemeral fluctuating temperatures between 27 °C and 35 °C, applied during 30 days around the critical period, were sufficient to significantly skew the sex ratios of *O. aureus* progenies, even though the masculinizing effect was relatively lower than those produced under constant high temperature

treatment. Similarly, Baroiller et al. (1995b) demonstrated that shorter exposures to masculinizing temperatures (*i.e.* 10 days instead of 30) or long exposure (30 days) to constant temperature slightly inferior to the supposed temperature threshold of maximal thermal influence (from 36 to 32 °C) could similarly skew the sex ratios of progenies of Nile tilapia in favour of males. These data are in support of the hypothesis that environmental conditions encountered by tilapia fry schools, at least in sub-tropical areas, might be sufficient to affect the gonadal sex differentiation in thermosensitive wild progenies.

At first glance, it might appear surprising that all the studied populations exhibited TSD regardless of their respective natural temperature regimes. Modifications of the balance between the components of sex determination system (genetic *versus* environmental) may be expected when populations are exposed to changing environmental conditions assuming differential expression of ESD. Especially under constant environmental conditions favouring one of the sexes through the generations, the Fisher's theorem (Fisher, 1930) predicts that ESD might be overridden by GSD, by giving an advantage to genotypes favouring the return to a balanced population sex ratio. Such a possibility of modification of sex determination systems has been shown experimentally in *M. menidia* (Lagomarsino and Conover, 1993). Conversely when the environment does not influence sex differentiation throughout generations, ESD would be either eliminated or conserved silently. Our results suggest that the evolution of complex sex determination systems, including both GSD and TSD, exposed to different environmental conditions acting differentially on the TSD component of the system, will not systematically tend to eliminate or even to reduce this component.

Even if a clear adaptive significance of TSD has only been demonstrated in a single fish species, *M. menidia* (Conover, 1984), a similar role cannot be definitively excluded for other thermosensitive species, especially at a minor or secondary level. The investigation of thermosensitivity directly in natural conditions and its impact on the individual and population fitness, especially in highly fluctuating systems, would be very interesting in order to shed light into the potentially adaptive significance of a complex and polymorphic system of sex determination in tilapias.

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