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Influence Of High Water Temperature On Sex Differentiation In An Ornamental Fish

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Abstract: ***Identification of sex in lower animals is a difficult task, since genetic and ecological factors are also involved in sex determination. Deciding the sex of a developing embryo has a paramount importance in genetic studies, which is a sensitive process. Therefore, sex- specific genetic markers can be developed using advanced molecular techniques such as RAPDs, AFLPs. Sex determination and differentiation in fish is a complex trait. But as fish are ectothermic in nature, it is known that water temperature can modify the sex determination.***

In this context the present study is aimed to observe the effect of short term exposure of 20/25/30 days post hatching (dph) juveniles of Zebra fish, Danio rerio (albino) exposed to high temperatures (32 ± 0.5°C/36 ± 0.5°C) on phenotypic sex with reference to genetic sex. 72 hrs exposure to elevated water temperature was found to exhibit phenotypic sex ratio of ♂:♀ of 68:22/56:44 (in 20/25dph respectively) at 32 ± 0.5°C and 98:2.0/ 72:28 (in 20/25dph respectively) at 36 ± 0.5°C compared to 50:50 ratio in control group maintained at 28±0.5° C or those exposed to higher temperatures at 30dph. Impact of high temperature on sex differentiation is confirmed compared to genotypic ratio predetermined through sex-specific RAPD markers. This study clearly showed that early juveniles subjected to temperature increase by 4-8 ° C even for 72 hrs will undergo variation in sex differentiation resulting to the generation of 18 % to 48% more males and thus leading to significant variation in sex ratio in population which may in turn lead to greater ecological imbalance.

Keywords: Zebra fish, phenotypic markers, Sex differentiation, RAPD markers.

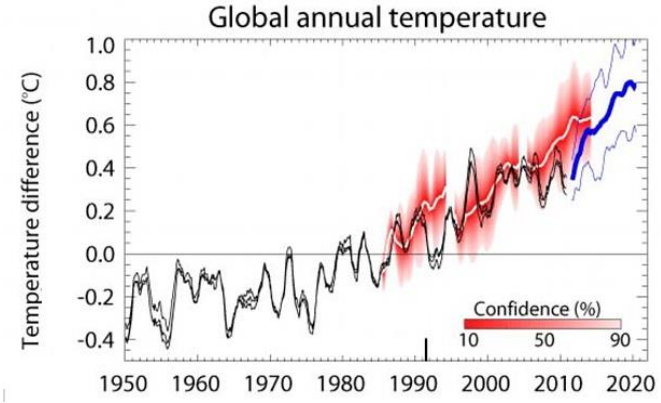
I. INTRODUCTION

Fishes are known abundant vertebrates on earth that exhibit a large variety of mechanisms of sexual determination. Sexual determination was referred to the event that compromises a bipotential gonad to develop as an ovary or a testicle (Hayes *et al.*, 1998). The processes of sexual determination and differentiation in fish is a complex process and a labile mechanism, under the control of both genetic factors and certain environmental factors (Baroiller *et al.*, 1999; Munday *et al.*, 2006; White *et al.*, 2006; and Sandra *et al.*, 2010).

The mechanism that determines whether the bipotential gonad will differentiate as a testes or an ovary is one of the most plastic processes in the evolutionary developmental biology of fishes. (Herpin and Schartl, 2008).Many teleostean species do not present morphologically differentiated sex chromosomes and hence sexing is difficult and alternative techniques to those based on karyotyping are commonly used for the determination of sex. But since DNA structure is not anticipated to change with altered physiology or environments. (Felip *et al.*, 2005; Bardakci, *et al.*, 2000; and Kovacs *et al.*, 2001).RAPDs, RFLPs, AFLPs are considered as sex-specific DNA sequences.

Several environmental factors are known to trigger the process of gonad differentiation leading to altered sex ratio in wild as well as farmed fish (Siegfried, 2010). Further temperature is the predominant of all that examined to date

that significantly influences several biological aspects in fish (Fuiman *et al*., 1998; and Koumoundouros *et al*., 2001). Temperature dependent sex determination (TSD) was first identified in Atlantic silverside by Conover & Kynard in 1981. Since then TSD has been studied in several fish species so far with a great range of temperature response (Strussmann & Nakamura, 2002; Godwin *et al*., 2003, Ospina-Alvarez &Piferrer, 2008).



Environment Blog, Global annual temperature difference according to Met office, 2016.

Given the growing concern of global warming, it becomes imperative that studies related to the effect of temperature on fish sex determination need to be explored further. Thus the present study aimed to observe the impact of high water temperatures (32 ± 0.5°C/36 ± 0.5°C) during gonad transition period of juveniles on phenotypic sex ratio in Zebrafish, Danio rerio. Further the comparison is also made with genotypic ratio considering Zebrafish sex-specific RAPD markers established earlier in our laboratory.

II. MATERIALS AND METHODS

SELECTION AND MAINTENANCE OF TEST SPECIES

20 /25/30 days post hatchling juveniles and adult male and female D. rerio *albino* were obtained from southern Aqua farms, Chennai, India and maintained in the laboratory in concrete tanks of 50L capacity at a stocking density of 100 nos. Prior to use the water was aerated overnight. Adult fish were acclimated to 28±0.5 ºC, fed on live tubifex worms and a basal diet 3 times a day and maintained with a photoperiod of 14h: 10h, light: dark regimen. while the post hatchling juveniles were fed on newly hatched brine shrimp.

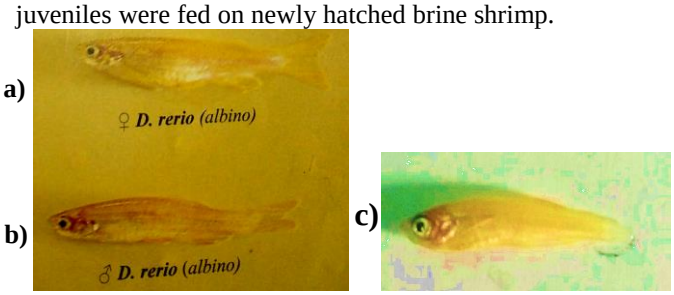


Figure 1: Adult a) female b) male, and c) 15 days post hatchling (dph) juvenile of Zebrafish, (D. rerio albino)

TEMPERATURE TREATMENT

900 nos. of 15 days post hatchling (dph) juvenile D. rerio were maintained at 28 ± 0.5˚C for 10 hrs. and divided into three groups of 300 Nos each. The recirculation system was equipped with a heating system (Biotherm) to keep the temperature at the required level. Water temperature was monitored at regular intervals during the experimental period. First group of juveniles of 300 nos. were subdivided into three groups of 100 each and transferred to water baths preheated to 28/32/36 ± 0.5˚C and maintained for 3 days in order to obtain 20 dph. The second groups of 300 nos. were also subdivided into three equal groups and maintained at 28 ± 0.5˚for 5 days and for 3 days at 28 /32/36 ± 0.5˚Cto obtain 25 dph stage. Similarly, the third group of 300 nos. were also subdivided into three equal groups and maintained for 10 days and further exposed to 28 /32/36 ± 0.5˚C to obtain 30 dph stage. All the groups maintained at 28 ± 0.5°C served as controls and all the groups were reared to adult stage for the assessment of phenotypic or genotypic markers.

PHENOTYPIC MARKERS: Healthy adult male and female D.rerio (*albino*) at the age of three months approximately was selected for the assessment of phenotypic markers following Kottelat and Vidthayanon (1993). Body color, eye color, pigmentation, width of abdomen, total body length, standard length, body depth, base length (mm) and number of dorsal, pectoral, anal and caudal fin rays were considered to assess sexual difference between male and female fish.

III. ASSESSMENT OF GENOTYPIC MARKERS

DNA EXTRACTION: DNA of adult male/female or juveniles was extracted following the Phenol/chloroform method following Ash Burner (1989). DNA samples were washed twice with the buffered phenol and twice with the mixture of Phenol: Chloroform: Isoamyl alcohol (25:24:1) and precipitated adding 1/10th volume of 2.5M Sodium Acetate (P^H5.8) and two volumes of ice cold ethanol. DNA samples were qualitatively assessed at 260 and 280 nm following Glasel (1995). The ratio of the OD readings at 260 and 280 nm is considered to give an estimate of the purity of the nucleic acid. Only samples of high purity (1.8 to 2.0) were considered for subsequent use. Genomic DNA was subjected to RAPD - PCR using female sex specific random primer OPA 12 (5'-TCGGCGATAG -3') using the following PCR conditions.

Steps	Temperature	Time	Cycles
Initial step denaturation	94°C	2 min	1
Denaturation	94°C	1 min	45
Annealing	36°C	1 min	
Extension	72°C	2 min	
Elongated extension	72°C	5 min	1
Soak	4°C	-	

Table 1: Conditions for RAPD, PCR

ANALYSIS OF RAPD-PCR PRODUCTS

Aliquots of each PCR reaction were separated through 1% agarose gel electrophoresis. 25µl aliquot of each PCR reaction was loaded on 1% agarose gel in 1% TBE (0.089M Tris base, 0.089M Boric acid, 0.02 M EDTA) containing 1µg/ml of ethidium bromide. Electrophoresis was performed at 80V for 1 hr. Gels were observed and the analysis has been carried out using Gel documentation system and Analyzer (Alpha Innotech).

STATISTICAL ANALYSIS: Results of each parameter was expressed as Mean ± S.D of 5 individual observations .The significance between the control and experimental groups was assessed through Student t-test at $p \leq 0.05$ level.

IV. RESULTS & DISCUSSION

Observations related to male Vs female D.rerio (*albino)* was shown in Table 1.

Phenotypic Feature	♂ Danio rerio (*albino)*	♀ Danio rerio (*albino)*
Body color	Albino*	Albino*
Longitudinal Stripes	Yellow*	Yellow*
Pigmentation	Absent*	Absent
Eye color	Albino^{+}	Albino^{+}
Total body Length (mm)	31.6 ± 1.15	24 ± 1.15
Standard Length (mm)	25.4 ± 0.87	21.2 ± 1.15
Head Length (mm)	6.36 ± 0.15*	6.12 ± 0.1*
Body Depth (mm)	8.12 ± 0.51	9.73 ± 0.43
Base Length of dorsal fin (mm)	4.13 ± 0.15	2.97 ± 0.43
Number of Dorsal fin rays	12.0 ± 1.0	8.0 ± 2.0
Number of Pectoral fin rays	13.0 ± 1.0	10.2 ± 1.0
Number of Anal fin rays	8.0 ± 2.0	4.0 ± 1.0
Number of Caudal fin rays	16.0 ± 2.0	12 ± 2.0

Table 1: Phenotypic features of male and female D. rerio (albino)

Significant sex-specific differences were noticed in total body length, standard length, body depth, base length and number of dorsal, pectoral, anal and caudal fin rays. Hence these features are selected as sex-specific phenotypic markers for the assessment of male and female ratio in experimental groups.

Wang *et.al*, (2007) and Takahashi *et.al*, (1997) stated that during the gonad transition period (20-30 dph) some of the undifferentiated gonads develop into an ovary-like gonad and become ovaries otherwise some of the oocytes develop into testes while the remaining oocytes undergo apoptosis. Sexual differentiation in Zebrafish is known to occur after hatching and is a labile process. Therefore, Zebrafish are particularly susceptible to factors in the environment that may modify or interfere with this process. 20 and 25 dph juveniles showed Male: Female differentiation of 68:32 and 59:41 respectively upon exposure to 32º C and the same developed into 98:2 and 72:28 respectively upon exposure to 36ºC (Table 2) compared to the controls maintained at 28 ± 0.5ºC that showed 50:50 male: female differentiation. This clearly indicated that elevation of water temperatures even by 4ºC can also influence the sex determination and lead to more male progeny production. Exposure of 30 days post hatching (dph) juveniles to 32ºC or 36ºC did not cause significant change in sex ratio compared to the controls.

As the elevated water temperatures have a significant effect on phenotypic sex ratio during the development and 25-35 dph was represented as the earliest thermo-sensitive window for sex determination in Zebrafish. But in the present study it is clear that exposure of 17 /22/27dph juvenile to high temperature for 72 hrs can influence massive degeneration of oocytes or apoptosis of ovaries and differentiation of gonads into testis leading to change in sex ratio biased more to male production (Table 2).

S. No	Group	Sub group	Male: Female
1.	28 ± 0.5 ºC	20 dph	50:50
		25 dph	52:48
		30 dph	49:51
2.	32 ± 0.5 ºC	20 dph	68:32
		25 dph	56:44
		30 dph	48:52
3.	36 ± 0.5 ºC	20 dph	98:2.0
		25 dph	72:28
		30 dph	53:47

Table 2: Phenotypic ratio of adult males: females of 20/25/30dph juveniles exposed to 32/36 ± 0.5 ºC for 72 hrs compared to the controls reared at 28 ± 0.5 ºC till maturation

Amplification of genomic DNA (Fig 2) with female sex-specific RAPD marker established (Fig 3) using OPA 12(5'-TCGGCGATAG -3') represented three bands of molecular mass Viz., 2920 bp, 2248bp, 1249bp (Fig 4) similarly RAPD analysis of experimental groups further clearly showed that 18 out of 68 % (20 dph group) and19 out of 59 % (25 dph group) generated at 32 ± 0.5 ºC (4: 6 male : female genotypic ratio) and 48 out of 98 % (20 dph group) and 22 out of 72 % (25 dph group) generated at 36 ± 0.5 ºC (5:5 male : female) (Fig 5) are originally genetic females but differentiated to phenotypic males.

Hence it is demonstrated that exposure of fishes to elevated temperatures which is common in the current scenario of global warming may lead to significant imbalance in sex ratio and male dominated fish populations with more ecological effects on biodiversity.

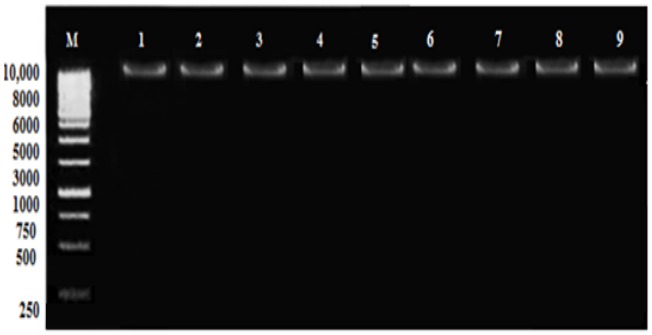


Figure 2: Genomic DNA of D.rerio (albino) maintained at 28 ± 0.5° C (Lane 1-3) and temperature treated group at 32 ± 0.5° C (Lane 4-6) and 36 ± 0.5°C (Lane 7-9). (M-1Kb ladder)

Figure 3: Electrophorogram of RAPD amplified products of individual adult male (Lane 1 – 6) & female (Lane 7-10) Danio rerio (albino) using primer OPA 12 (M-1Kb ladder

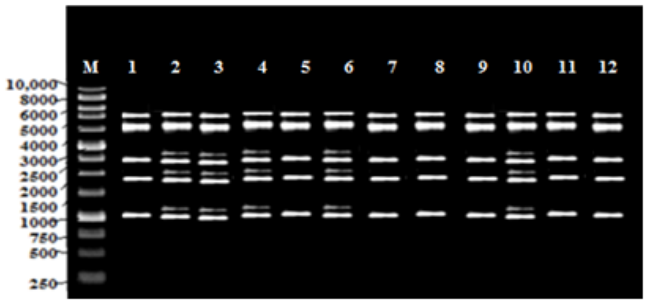


Figure 4: Electrophorogram of RAPD amplified products using primer OPA 12 of individual adult male (Lane 1) and female (Lane 2) D.rerio maintained at 28 ± 0.5˚C (controls) and 20 dph (Lane 3-7) and 25 dph (Lane 8-12) grown to maturity (at 28 ± 0.5 ºC) exposed to (32 ± 0.5°C) for 3 days. (M-1Kb ladder)

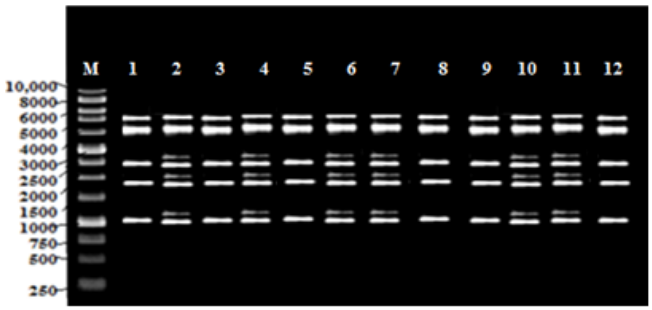


Figure 5: Electrophorogram of RAPD amplified products using primer OPA 12 of individual adult male (Lane 1) and female (Lane 2) D.rerio maintained at 28 ± 0.5˚C (controls) and 20 dph (Lane 3-7) and 25 dph (Lane 8-12) grown to maturity (at 28 ± 0.5 ºC) exposed to (36 ± 0.5°C) for 3 days. (M-1Kb ladder)

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