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Temperature during early life determines sex in zebrafish, *Danio rerio* (Hamilton, 1822)

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It is well established that phenotypic sex in many gonochoristic fish species is the combined outcome of genetic and environmental factors, with temperature having the most profound influence of any environmental factor on sex differentiation. This study demonstrates that water temperature during early life (from spawning up until after metamorphosis) has a drastic influence on the sex ratio of zebrafish (*Danio rerio*), with male-biased populations produced at lower temperatures (22°C, 87.1% males) and female-biased ones at higher temperatures (31°C, 82.4% females). Since zebrafish is employed extensively as a model organism for a variety of research studies, these results can be of great importance to the designing of experiments where the sex of the fish is of relevance to the studied parameters.

Key words: temperature, zebrafish, larval rearing, sex differentiation.

INTRODUCTION

Of all environmental factors examined to date in poikilothermic vertebrates, temperature exerts the most significant influence on all aspects of biology in fishes (Conover & Kynard, 1981; Policansky, 1982; Seikai *et al*., 1986; Polo *et al*., 1991; Blaxter, 1992; Fuiman *et al*., 1998; Koumoundouros *et al*., 2001). Thus, given the growing concern of global warming, it is understandable that studies on the effect of temperature on fish physiology and ecology are given increasing attention recently (Ospina-Alvarez & Piferrer, 2008; Beaugrand & Kirby, 2010).

Unlike higher vertebrates, where gonochorism and strong genetic channeling of the sexual development are the rule, fishes include species that reproduce through gonochoristic, hermaphroditic (sequential or simultaneous) and parthenogenetic mechanisms (Yamamoto, 1969; Strüssmann & Nakamura, 2002). Even among gonochoristic fishes, the ultimate fate of the developing gonads may be decided by a delicate equilibrium of genetic and environmental (physical, chemical, or social) factors, so that often the phenotypic sex does not conform to the genotypic sex (Baroiller *et al*., 1999; Strüssmann & Nakamura, 2002). As a result, a simple unifying model to explain the genetic basis of sex determination in fishes does not exist today (Baroiller *et al*., 1999; Devlin & Nagahama, 2002). Of all the examined environmental factors (e.g., temperature, stocking density, pH), temperature seems to be the main environmental determinant of sex (Baroiller *et al*., 1999), in a process called temperature-dependent sex determination (TSD). Temperature sex determination in fish was first identified in the Atlantic silverside (*Menidia menidia*), where high or low temperatures were shown to lead to female- or male-biased populations, respectively (Conover & Kynard, 1981). Since then, TSD has been documented in several fish species with a great range of temperature responses (Baroiller *et al*., 1999; Devlin & Nagahama, 2002; Strüssmann & Nakamura, 2002; Godwin *et al*., 2003, Ospina-Alvarez & Piferrer, 2008). Sex differentiation in fishes is regulated by sex steroid hormones, which in turn are controlled by various genes controlling steroidogenic enzymes (Baroiller *et al*., 1999). The existence of TSD is due to

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the disruption of steroidogenesis in early gonad formation, through the modulation of aromatase gene expression (Strüssmann & Nakamura, 2002; Godwin *et al*., 2003). On the other hand, it is also established nowadays that the environmental temperature effect on the sex ratio of a species with identified sex chromosomes is a strong indicative of genotypic sex determination (GSD – Devlin & Nagahama, 2002; Ospina-Alvarez & Piferrer, 2008) rather than TSD.

Zebrafish (*Danio rerio*) is a valuable model-organism for many research areas and is used widely by laboratories throughout the world (Lawrence, 2007). Oddly enough, the effect of the environment, and especially temperature, on this species' development has not been studied thoroughly (Sfakianakis *et al.*, 2011), and very little is known concerning the influence of temperature on sex differentiation (Uchida *et al*., 2004; Ospina-Alvarez & Piferrer, 2008). Zebrafish is an undifferentiated gonochoristic species, which goes through a phase of juvenile hermaphroditism (Takahashi, 1977). At 10-12 days post fertilization (dpf) gonads begin to differentiate into ovaries, irrespective of the individual's genotypic sex. Ovaries continue to develop until 23-25 dpf, after which time testicular differentiation begins in genotypic males. Ovarian development continues further in the females, while in the males the ovaries degenerate simultaneously with testicular development (Takahashi, 1977). Currently, zebrafish are reared at different temperatures –within their thermal range– in laboratories around the world, without any attention to the effect that differences in rearing temperature may have on the sex ratio of the population, which in turn may have significant influences on the studied parameters (e.g., growth, metabolic rates, ontogenetic rates, gene expression). In the present study, the effect of rearing temperature during early life on sex differentiation of zebrafish was examined.

MATERIALS AND METHODS

Rearing

Zebrafish eggs were obtained from wild type broodstock (ZF WT2 F5, Wageningen Agricultural University, The Netherlands) maintained at a sex ratio of 2:1, females:males (a total of 45 individuals), at the facilities of the University of Crete, Greece. The fish were fed three times daily with industrial dry food (Sera Vipan, Germany), three times weekly with newly hatched *Artemia* sp. nauplii (Instar I, INVE SA, Belgium) and their temperature was regulated at 28˚C. Spawned eggs were collected and counted under a stereoscope (Olympus, SZX9) and submerged for 3-5 min in methylene blue solution (0.001%) for anti-fungal protection (Westerfield, 1995). Three hundred eggs were then placed in 2.6 l rearing cages inside 120 l tanks maintained at different water temperature each time (22˚C, 25˚C, 28˚C and 31˚C) and after the appropriate acclimation process (10 min per degree of temperature change). Each temperature was kept stable through the combined use of thermostat heaters and coolant devices. All eggs used (1200 divided to 4 treatments), came from a single spawning and all temperature treatments were performed in duplicate. In total, 2400 eggs were used in the present study. To simulate conditions in natural habitats of the species, the different temperature regimes were applied during the entire course of the experiment, until complete sex differentiation, rather than targeting a specific developmental stage(s). The hatched larvae were fed three times daily (*ad libitum*) with *Paramecium* sp. (Blades Biological CO, UK) followed by newly hatched *Artemia* sp. nauplii. At the onset of *Artemia* sp. feeding, the larvae were released in the 120 l tank. Throughout the rearing phase there was constant monitoring and adjustment of temperature and pH, while oxygen saturation was kept above 80%. Rearing was done under 14L:10D artificial photoperiod.

Fish sampling

Rearing ended after metamorphosis at 107-94 days post hatching (dph), when fish had a mean total length of 29.2 ± 2.6 – 34.0 ± 2.3 mm (mean ± s.d.) depending on rearing temperature. From each duplicate cage, 25 fish were collected randomly, euthanized with ethylenglycol-monophenylether (Merck, 0.2 – 0.5 ml l^{-1}), and fixed in 4% formaldehyde and 1% gluteraldehyde (McDowell & Trump, 1976). Prior to embedding, the head and tail of each specimen was cut and the body trunks were dehydrated in a 70-95% ethanol series. For embedding in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany) body trunks were cut in half along their anterior-posterior axis, and the two pieces were placed in the same block so that sectioning proceeded in a medio-posterior direction for one piece and in a medio-anterior direction for the other. Serial sections were obtained at a thickness of 3-5 μm on a microtome (Biocut 2035, Reichert Jung, Germany) using disposable blades. After drying, slides were stained with methylene blue/azure

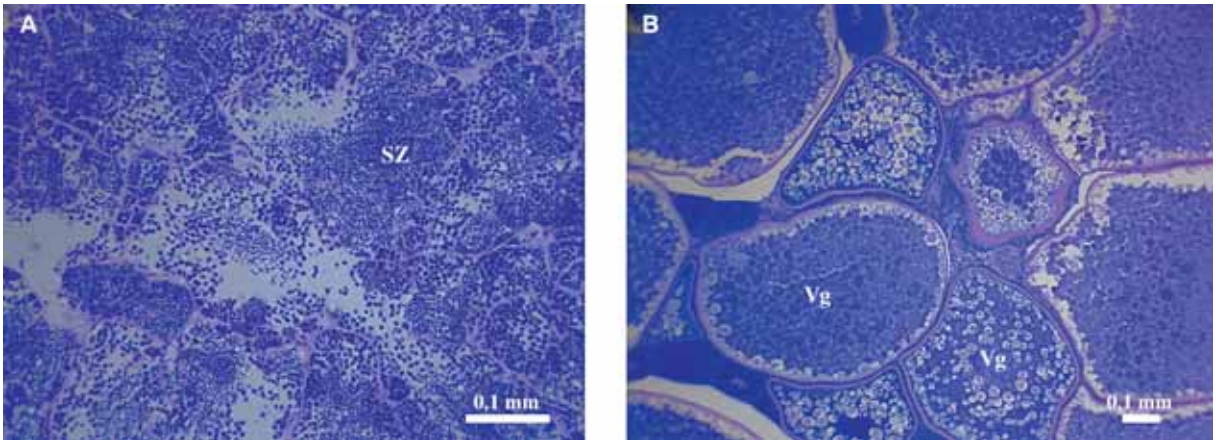

FIG. 1. Histological sections of zebrafish gonads. A. Mature zebrafish testis at spermiation, filled with spermatozoa (sz). B. Zebrafish ovary filled with vitellogenic oocytes (Vg).

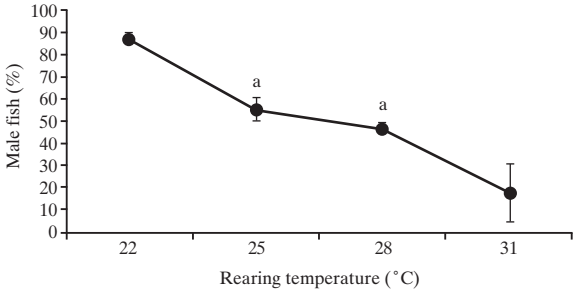

FIG. 2. Percentage of male individuals in the zebrafish populations (n = 2) reared at different temperatures. The bars represent means ± s.d. The superscript "a" indicates sex ratios that did not differ significantly (G-statistic; $p > 0.05$) from a 1:1 sex ratio.

II/basic fuchsin (Bennett *et al*., 1976) and examined under a light microscope (Nikon Eclipse 50i, Japan) for the presence of a testes or ovary.

Laboratory fish were handled according to the European Union Directive (86/609 EEC) for the protection of vertebrate animals used for experimental and other scientific purposes (EEC, 1986).

Statistical analysis

The replicated goodness of fit test (G-statistic; Sokal & Rohlf, 1995) was used to compare survival rates, sex ratios between treatments, and sex ratios between treatments and a theoretical 1:1 sex ratio. Statistical significance was accepted at $p \leq 0.05$.

RESULTS

The survival rates between the different experimental groups were similar (88-92%, $p > 0.05$). Histological observation of the gonads (Fig. 1) revealed that lower temperatures induced masculinization whereas higher temperatures resulted in female-biased populations (Fig. 2). The populations reared at 22°C and 31°C differed significantly ($p < 0.01$), at opposite sides, when compared to the theoretical 1:1 ratio.

DISCUSSION

The sex ratio shift of zebrafish from male to female dominance as rearing temperature increased from 22°C to 31°C demonstrates the importance of TSD in this very important model-organism. This statement, although positively documented through experiments, tends to be controversial mainly because a series of new studies argue that the proof of the existence of TSD in a fish species should be accompanied by certain facts.

For example, Ospina-Alvarez & Piferrer (2008) using a slight modification of the criteria of Valenzuela *et al*. (2003) and Conover (2004) suggested recently that in order for a fish species to be considered to have TSD, the following two conditions must apply: i) absence of sex chromosomes, and ii) sex ratio shifts must occur within the range of temperatures applying during development (RTD) and not within the range of natural temperature (RNT) where the

species usually live in (and which is usually much broader). Although sex chromosomes in zebrafish have not yet been identified, the inheritance pattern of genetic all-females used in the study of Uchida *et al*. (2002), suggest that the genetic sex of zebrafish is determined by an XY sex chromosome system. Therefore, based on the arguments of Ospina-Alvarez & Piferrer (2008), it appears that the sex determining mechanism in zebrafish is not temperature (TSD) but genotypic dependent (GSD) and that the observed sex ratio shifts might be the consequence of the thermal effect (TE) on GSD (GSD + TE).

Regarding the classification of Ospina-Alvarez & Piferrer (2008), it is interesting to note that as far as the second criterion is concerned, there are different opinions as to how exactly the RTD is defined in zebrafish. Ospina-Alvarez & Piferrer (2008) cite Froese & Pauly (2008) that report the RTD of zebrafish being between 26˚C and 29˚C. On the other hand, recent studies (Engeszer *et al*., 2007; Spence *et al*., 2008) advocate that the RTD in zebrafish is at high temperatures and can range from 27˚C up to 34˚C. If so, then the results of the present study show definitely that there is a sex ratio shift inside the RTD (from 50% males at 28˚C to almost 17% at 31˚C).

The effect of rearing temperature on sex differentiation in zebrafish has been studied before by Uchida *et al*. (2004), which treated genetic all-female juveniles with very high temperatures and reported that the percentage of gonadal masculinization at 28.5˚C, 35˚C and 37˚C were 0%, 68.8% and 100%, respectively. But when the same authors treated wild-type zebrafish juveniles with the same temperature regimes (between 15 and 25 days post-hatching), they did not observe any change in the sex proportion of the populations (unpublished data as stated in Uchida *et al*., 2004). The present study seems to contradict the findings of the work of Uchida *et al*. (2004), but the temperatures used by these authors are considered quite extreme for the species since normal development above 34˚C is not possible under aquaculture conditions (Schirone & Gross, 1968). Another major difference in the experimental design of the two studies that could account for the different results is the time window used for the application of temperature. Shang *et al*. (2006) proposed that the thermo-sensitive period for sex determination in zebrafish is from 10 to 42 dph. It is possible then that the time window used by Uchida *et al*. (2004) (15 to 25 dph) did not include the entire thermo-sensitive period of the species and therefore did not allow for the full effect of temperature. Finally, it should be noted that among the patterns of sex ratio response to temperature that have been demonstrated by Ospina-Alvarez & Piferrer (2008), zebrafish-as shown in the present study-clearly belongs to the second pattern (low temperatures produce male-biased sex ratios and high temperatures produce female-biased sex ratios), thus being the only fish species studied so far that follows exactly that pattern.

The effect of food abundance, and therefore growth rate, on the sex ratio of zebrafish juveniles has also been studied recently (Lawrence *et al.*, 2008). The outcome of that study was that faster-growing zebrafish were more likely to become females than their siblings that were fed less and, therefore, grew at a much slower rate. This is in accordance with the present findings, since elevated developmental temperatures in zebrafish [within the recommended range for culture (Schirone & Gross, 1968; Matthews *et al*., 2002)] resulted in higher growth rates (personal observations; Lawrence, 2007). Lawrence *et al*. (2008) also suggested that growth rates are the guiding environmental factor for sexual differentiation in zebrafish and that this could be the case in any variant of environmental sex differentiation that involves sex-specific differences in growth rates.

Considering the patterns of TSD in reptiles, it was suspected that a pivotal temperature exists, which is defined as the temperature which gives 50% of individuals of each sexual phenotype (Pieau, 1996). Since zebrafish also presents oppositely distorted sex ratios at lower and higher temperatures, based on the present findings it is assumed that the pivotal temperature of zebrafish is between 25˚C and 28˚C. Similarly, in earlier studies with pejerrey (*Odontesthes bonariensis*) (Strüssmann *et al*., 1996, 1997), it was found that the female proportion decreased from 100% at 19˚C to 0% at 29˚C with intermediate temperatures producing populations with equal proportion of the two sexes. In zebrafish here, there was also a seemingly linear response of sex ratios to rearing temperature, albeit in an opposite way to the pejerrey. This raises the possibility that if a wider thermal spectrum is examined, monosex populations at the opposite ends of the temperature range may be produced in zebrafish as well. Projection of a linear regression analysis of the present data reveals that the theoretical 100% and 0% male populations may be achieved at 20˚C and 34˚C, respectively. The results obtained in the present study can be of great relevance to numerous studies using zebrafish as a model-organism

(Key & Devine, 2003), allowing the avoidance or production of sex-biased populations.

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