Environ Biol Fish
DOI 10.1007/s10641-007-9195-8

FULL PAPER

Rapid growth and out-crossing promote female development in zebrafish (*Danio rerio*)

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Received: 15 October 2006 / Accepted: 11 December 2006

Abstract Sex determination in fishes is often enigmatic, a situation that is often made even more complex by the fact that the process of sexual differentiation in many species may be influenced by environmental conditions. This situation is typified in zebrafish, a popular model organism. Despite the vast array of information available for the species, the genetic controls of sex are unknown. Further, environmental parameters, such as rearing densities, seem to exert an influence on the sex ratios of captive stocks. In an effort to dissect the genetic and environmental controls underlying the expression of sex in this species, we manipulated growth of pure-bred and out-crossed zebrafish by varying their food supply during development. Faster-growing zebrafish were more likely to be female than siblings that were fed less, and out-crossed broods had higher proportions of females than broods from pure-bred crosses. The dependence of sex ratio on feeding rate is readily understood in terms of adaptive sex allocation: zebrafish life history seems to confer the greater pay-off for large size on females. A similar male/female difference in the pay-off for hybrid vigor could similarly account for the female bias of out-crossed broods—and it could be a manifestation of Haldane's rule.

Keywords Environmental sex determination · *Danio rerio* · Haldane's rule · Food consumption · Size

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Introduction

One of the hallmarks of sex determination in teleost fishes is that the process of primary sexual differentiation may often be influenced by environmental conditions during development. The most numerous and best characterized examples of so-called environmental sex differentiation in fishes involve rearing temperature, but pH, social interactions, and population density are also frequently cited in reviews of the subject (Godwin et al. 2003). Of these, sex differentiation through social interaction and density dependence is perhaps the least understood.

Density-dependent sex differentiation has been demonstrated in two fishes, and in both instances density also influenced growth. In paradise fish, *Macropodus opercularis*, the proportion of males

in broods is inversely correlated with density, and fish reared in isolation grow more rapidly and usually develop as males (Francis 1984). Crowding during development reduces growth rates and promotes male development in both natural and captive populations of American eels, *Anguilla rostrata*, while low population densities produce higher rates of growth and a predominance of females (Krueger and Oliveira 1999). Francis and Barlow (1993) conjectured that dominance interactions involving access to resources mediate growth rates and influence sexual differentiation in Midas cichlids, *Cichlasoma citrinellum*. Individuals that grow more rapidly become male, while individuals that grow more slowly tend to differentiate as females.

Growth rates in many fish species are dependent on density or social status (Lorenzen and Enberg 2002), with individuals of high status or in low-density populations growing more rapidly than individuals of low status or in high-density populations. In both American eels and paradise fish, rearing densities may mediate rates of growth that in turn affect sexual differentiation—and in the case of the Midas cichlid, behavior mediates growth. It may be growth, and not density or social interactions, per se, that influences sexual differentiation in species exhibiting both density-dependent and socially controlled sex differentiation.

We have tested this hypothesis in zebrafish, *Danio rerio*, a small freshwater cyprinid native to South Asia that has become a popular developmental and genetic model (Fishman 2001). The mode of genetic sex determination in this species is unknown—no sex-determining gene has been identified and none of the 25 chromosome pairs are heteromorphic or display sex linkage (Traut and Winking 2001). However, researchers using standard lines of zebrafish that have long been maintained in laboratories are often plagued by severe sex ratio distortions, suggesting that selection or inbreeding may influence sex differentiation. On the other hand, Uchida et al. (2004) were able to produce highly male-biased broods by exposing fish to high temperatures during development, and anecdotal evidence from zebrafish culture facilities indicates that rearing zebrafish at low densities favors female development, while high densities promote male development (Nusslein-Volhard and Dahm 2002). These observations imply that environment and genetic background both influence sex determination in zebrafish. To examine the relationships between growth, genetic background, and sexual differentiation, we manipulated growth rates in pure-bred and out-crossed zebrafish by varying the amount and frequency of feeding in treatment groups of equal densities.

Material and methods

Two genetically distinct laboratory strains of zebrafish, Tuebingen (TU) and WIK, were used to generate four cross types. Tuebingen is a laboratory strain that was screened to remove embryonic lethal mutations before being used for mutagenesis and sequencing of the zebrafish genome, and WIK is a mapping strain derived from a wild catch in India (www.zfin.org). Both have been maintained in laboratories for numerous generations by inbreeding. Broodstock for this study was maintained in 9 l tanks (~2 fish l^{-1}) on a 10,000 l recirculating system (20% water replacement per week), with temperature maintained at ~28°C, pH at 7.2–7.5, conductivity at 650–850 μS, and a 14-h light, 10-h dark photoperiod.

Four types of crosses were made by placing single males and females in mating cages on the evening prior to the day of spawning: TU male × TU female (TU/TU), WIK male × WIK female (WIK/WIK), TU male × WIK female (TU/WIK), and WIK male × TU female (WIK/TU). Each cross was replicated four times for a total of 16 matings. Spawning commenced the following morning, and fertilized eggs were collected 4 h after the lights came on, and placed in fresh water. Forty fertilized eggs from each of the 16 broods were divided into two treatment groups of 20 eggs each, transferred to individual Petri dishes containing fresh water, and placed in a 28°C incubator. The next morning (Day 1), two nonviable embryos (one in TU/WIK 1 ''high food'' and one in WIK/TU 4 ''low food'') were replaced with viable embryos from the same clutch. Fresh water was then added to each dish and embryos

were returned to 28°C incubators. At 5 days post-fertilization (dpf), the two treatment groups from each of the 16 broods were removed from the incubator and transferred to 2 l rearing tanks—one group to a ''high food'' tank, and the other to a ''low food'' tank.

Zebrafish in ''high food'' tanks received approximately twice as much food (a liquefied mixture of *Spirulina* powder and Hatchfry Encapsulon Grade 0 (Argent Chemical Laboratories), and 1st instar *Artemia* nauplii as those in ''low food'' tanks. Experimental feeding regimens were ended at 86 dpf; all fish were fed ''normal'' diet (*Artemia* 1st/2nd instar nauplii 2×, pellets 1× daily) for the duration of their lifespan.

Experimental tanks were placed on a recirculating water system at 5 dpf with a drip rate of 0.1 ml s^{-1}, increased to 2.0 ml s^{-1} after first *Artemia* feeding (6 dpf). At 36 dpf, all fish were transferred, for the last time, to 9 l tanks to minimize behavioral interactions that could affect growth. They remained in these tanks for the duration of the experiment.

At 5 dpf and every 7 days thereafter, random samples of five fish from each treatment group were briefly anesthetized with MS-222 (Aquatic Ecosystems), measured for total length under a dissecting microscope, and returned to their respective tanks. Growth rates of experimental groups were derived from the slopes of size versus age curves.

Adult zebrafish are sexually dimorphic and dichromatic; the sexes are clearly distinguishable upon sexual maturity in healthy fish maintained in laboratory conditions (Nusslein-Volhard and Dahm 2002). Adult zebrafish have five bluish-black stripes alternating with silver/yellow stripes. In females the non-black stripes are silver in color, while in males the non-black stripes are yellow. In addition, the bodies of mature females are filled with developing oocytes, making them less streamlined than mature males. Using these characters, phenotypic sex of fish in all treatment groups was assessed at 91 dpf and confirmed at 128 dpf. The reliability of visual sex scoring was verified at 100% by scoring and then dissecting a random sample of 50 adult fish from a general laboratory population. Of 538 experimental fish, six that could not be scored at the time of final confirmation were recorded as ''undifferentiated'' and excluded from all analysis. All of these fish were from TU/TU replicate group 4 (two individuals in the high food and four in the low food treatments, respectively), and were likely carriers of a viable recessive metabolic mutation (in which the animals essentially stop growing and never reproduce) that is commonly seen in this laboratory strain (personal observation).

Since portions of each brood were exposed to both high and to low food treatments, but each clutch represented only one of the four genetic crosses, two-way split-plot Analysis of Variance was used to analyze the effects of feeding regimen (2 levels) and cross type (4 levels) on survival, growth rate, and sex ratio. For growth rate and for sex ratio, we performed planned comparisons for three groupings of cross types: (1) pure-bred versus out-crossed, (2) WIK father versus TU father, and (3) WIK mother versus TU mother. To reduce heteroscedasticity, non-normality, and outliers, the angular transform was applied to growth rate metrics (slope of size–age curves), survival rate metrics (proportion survived), and sex ratio metrics (proportion female) before statistical analysis. Mean size (total length) of fish in high versus low food regimens, as well as the survival rates among treatment groups were compared using one-way ANOVA. The relationship between sex ratios and survival rates in treatment groups was analyzed using linear regression. Statistical procedures were performed using Systat, SPSS, and the analysis package on Microsoft Excel.

Results

Two treatment groups, TU/WIK 4A (high food), and WIK/TU 3A (high food) experienced mass die-offs early in the experimental period (days 15 and 22, respectively), with only two out of the original 20 fish surviving. In both cases, mortality was probably due to ammonia and/or hydrogen sulfide poisoning. These two groups, as well as their low-food replicates, TU/WIK 4B and WIK/TU 3B, were excluded from the analyses. Survival among the remaining 28 treatment groups was high, with a mean survival rate across all groups

of 0.88. Survival by cross type varied slightly, ranging from a low of 0.82 in TU/WIK to 0.95 in WIK/WIK crosses, but there was no significant difference in survival among crosses ($F = 1.945$, df = 3, $P = 0.186$). The difference between mean survival rates for high food (0.91) versus low food (0.86) groups was also not significant ($F = 0.934$, df = 1, $P = 0.357$), and there was no significant interaction between food treatment and cross type ($F = 1.491$, df = 3, $P = 0.276$). Linear regression indicates no relationship between sex ratio and survival rate (angular transform of both variables; $P = 0.798$, $r^2 = 0.003$).

Fish in high food treatments grew more rapidly than fish subjected to low food treatments (Table 1, Fig. 1). The mean slope of growth curves was 0.39 vs. 0.25 mm day^{-1} in high versus low food groups, and at the end of the experimental feeding period fish were larger in the high food groups (mean length = 32.74 mm) than in the low (mean length = 21.96 mm). Cross type also had a significant effect on growth rate (Table 1). However, planned post-hoc comparisons among levels of cross type revealed no significant effect on growth rates for out-crossed versus pure-bred crosses, crosses from WIK versus TU fathers, or crosses from WIK versus TU mothers. A non-significant interaction term indicates no interaction between cross type and food in their effects on growth rates (Table 1).

Sex ratios from each of the 28 analyzed treatment groups were highly variable, ranging from 25% to 100% female. As with growth rate, sex ratio was strongly influenced by experimental feeding regimen. The proportion of females in high-food treatment groups was higher than in low-food treatment groups (76% vs. 51% female; Table 1), with a higher proportion of females in high versus low food in all but 3 of 14 same-brood pairs, and with similar effects in all four cross types (Fig. 1).

Proportion of females in treatment groups also differed significantly according to cross type (Table 1). Planned post-hoc comparisons revealed that out-crossed treatment groups had a greater proportion of females than did pure-bred treatment groups (Table 1). Treatment groups derived from both the WIK/WIK and TU/TU pure-bred crosses had fewer females than males (44% and 45% female, respectively), whereas the out-crossed WIK/TU and TU/WIK groups were strongly female biased (93% and 84% female, respectively; Fig. 1). Indeed, four all-female groups arose from the out-crossed broods.

Although the increase in the proportion of females at high, compared to low, food levels was

Table 1 Analysis of variance of zebrafish growth rate and proportion female

Dependent variable	Growth rate				Proportion female			
	SS	df	F	*P*	SS	df	F	*P*
Tests of main effects								
FOOD	0.1551	1	442.216	0.000	0.6974	1	20.953	0.001
Error	0.0035	10			0.3328	10		
CROSS	0.0037	3	3.751	0.049	1.7757	3	7.422	0.007
Error	0.0033	10			0.7947	10		
FOOD*CROSS	0.0005	3	0.511	0.684	0.0458	3	0.458	0.717
Error	0.0035	10			0.3328	10		
Planned post-hoc comparisons of CROSS								
Purebred versus Hybrid	0.0000	1	0.002	0.969	1.6954	1	21.175	0.000
Error	0.1487	21.44			1.4826	18.52		
TU father versus WIK father	0.0011	1	0.154	0.698	0.0400	1	0.500	0.488
Error	0.1487	21.44			1.4826	18.52		
TU mother versus WIK mother	0.0021	1	0.303	0.588	0.0517	1	0.646	0.432
Error	0.1487	21.44			1.4826	18.52		

Two FOOD treatments crossed against four genetic CROSSes (two pure-bred and two out-crossed), and 14 Broods crossed against FOOD treatments and nested within CROSSes: tests of main effects; planned post-hoc comparisons among levels of CROSS

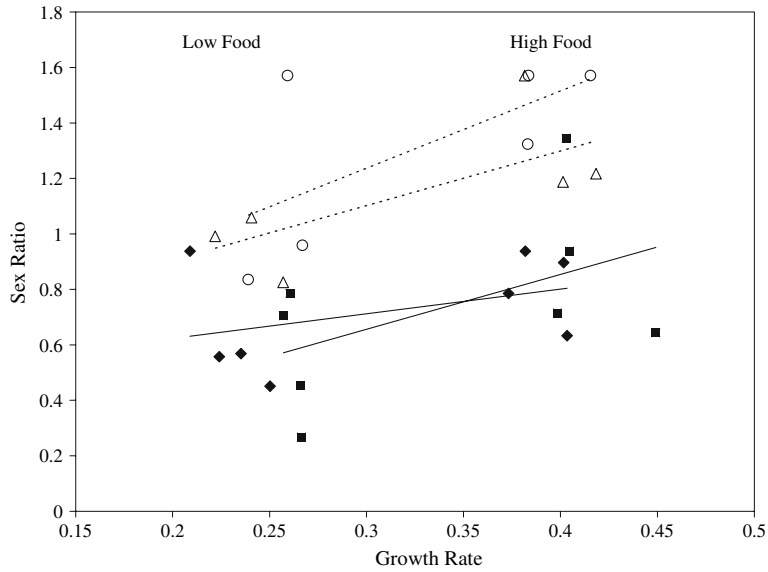

Fig. 1 Sex ratio (ArcSin {proportion female}$^{0.5}$) vs. Growth Rate (ArcSin {slope of body length vs. age }$^{0.5}$) for each of the 28 treatment groups. Sex ratio values range from 0 (all male) to 1.6 (all female). Growth rate for each treatment group was derived from the slope of the average growth curve. *Key*: Solid square, ■ = TU/TU pure-breds; solid diamond, ◆ = WIK/WIK pure-breds; open circle, ○ = TU/WIK out-crosses; open triangle, △ = WIK/TU out-crosses. The regression line is shown for each food/cross: dashed lines (and open symbols) for out-crosses, solid lines (and solid symbols) for pure-breds. The cluster of points with growth rates between 0.2 and 0.3 comprise all low food treatment groups, and points clustered between 0.35 and 0.5 comprise high food treatment groups

most pronounced in the TU/TU groups (+0.31) and was lowest in the WIK/TU groups (+0.17), the statistically insignificant interaction term in the two-way ANOVA indicated no interaction between the effects of cross type and feeding regimen on sex ratio (Table 1).

Discussion

The causal relationship between sexual differentiation and rearing density or social dominance in fishes has been unclear. Although growth rates could have influenced differentiation in some studies—due to effects of density in American eels (Krueger and Oliveira 1999) and paradise fish (Francis 1984), and effects of dominance interactions in Midas cichlids (Francis and Barlow 1993)—a direct link between growth and differentiation has not been demonstrated. In the present study, simply varying the frequency and amount of feed prior to and throughout the period of gonadal differentiation had a strong effect on sex ratios—due to the markedly different growth rates of high food versus low food treatment groups.

Our data lends support to the hypothesis advanced by Kraak and de Looze (1993) that any environmental influence on growth during a critical period of development is sex-determining. Differential rates of growth between the sexes have been observed in a number of fishes with ESD, including the paradise fish, American eel, Midas cichlid, European sea bass, *Dicentrarchus labrax*, and the Atlantic silverside, *Menidia menidia* (Conover and Kynard 1981; Francis 1984; Francis and Barlow 1993; Blazquez et al. 1999; Krueger and Oliveria 1999). However, in all of these instances, it is another environmental factor (density, social interactions, and temperature) that is presumed to be the determining factor for sexual differentiation, even though the process seems to be tightly linked with growth rates in all of these species. When we directly manipulated growth in zebrafish, a species that shows similar patterns to those listed above, we were able to affect the expression of phenotypic sex. Because our experimental design excluded the

influence of other environmental factors, our results provide strong evidence that growth rates are the guiding environmental factor for sexual differentiation for zebrafish. We suggest that this may also be the case in any variant of environmental sex differentiation that involves sex-specific differences in growth rates, but this remains to be tested.

The mechanism by which growth influences the expression of phenotypic sex in zebrafish is unclear. Gonadal differentiation in zebrafish is influenced by exogenous steroid hormones and by environmental stimuli such as temperature and density during a ''sensitive period'' of development (Kitano et al. 1999; Fujioka 2001; Piferrer 2001; Anderson et al. 2003; Uchida et al. 2004). Elevated temperatures block the production of cytochrome P450 aromatase, the enzyme responsible for converting endogenous androgens into estradiol (Kitano et al. 1999). As demonstrated in several fishes, including zebrafish (Uchida et al. 2004), exposure to high temperatures during the sensitive period results in overproduction of males, presumably because estradiol production is not adequate for ovarian differentiation (Piferrer et al. 1994). Growth rates in zebrafish may also affect the production of steroid hormones, since growth in fishes is regulated by complex interactions of several hormones, including gonadal sex steroids, and a positive feedback loop between synthesis of growth hormone and both testosterone and estradiol has been demonstrated in a number of fishes (Holloway and Leatherland 1998). In our experiment, growth rate during the sensitive period, which varied with nutritional status, may have influenced the production of growth hormone and other metabolic regulators, indirectly affecting steroid production and sexual differentiation.

Growth, rather than temperature, may be the environmental factor that most commonly affects sexual differentiation of zebrafish in culture. The high temperatures (>35°C) that produced male-biased broods in experiments (Uchida et al. 2004) are unlikely to occur in rearing facilities, but variation in zebrafish growth rate associated with densities and/or behavioral interactions during the sensitive period is common.

Could growth-dependent sex differentiation be adaptive in nature? Charnov and Bull (1977) hypothesized that environmental sex determination is adaptive when resources are patchy and have a gender-dependent effect on fitness. Cooler temperatures promoting female development and warmer temperatures promoting male development in Atlantic silversides (Conover and Kynard 1981) support this hypothesis. Since cooler waters occur early in the growing season, females have a longer growing period and so achieve a larger size than do the males that develop in the warmer waters that occur later in the growing season. Fecundity is strongly limited by body size in females because larger females produce more eggs than smaller females. Fecundity of males is less dependent on body size because silversides are group spawners, so individual males do not typically monopolize mating opportunities (Conover and Kynard 1981). Thus, the fitness benefit of large body size is greater for females than for males.

Growth-dependent sex differentiation may maximize reproduction in zebrafish in the same way. The zebrafish environment is patchy in time and space, as they live in a wide variety of habitats in the Indian subcontinent, ranging from stagnant rice paddies and ditches to larger streams and rivers (Rahman 1989; Talwar and Jhingran 1991; Menon 1999), and the monsoon climate has pronounced rainy and dry seasons. In addition, zebrafish are a shoaling species (Pritchard et al. 2001), and while males are territorial and reproductive success may be positively correlated to large body size (Pyron 2003; Spence and Smith 2004), body size probably has a more profound effect on the fecundity of females because larger females produce more gametes than smaller females. As in the Atlantic silverside, zebrafish individuals that grow slowly are more likely to have higher reproductive success as small males than as small females and individuals that are able to grow rapidly are more likely to have high reproductive success as large females.

Environmental stimuli and genotype interact to determine sex in some fishes. In Atlantic silversides, for instance, sex differentiation is strongly influenced by temperature in southern populations, while northern populations show little or no response to temperature (Conover and Heins 1987), and sensitivity to temperature varies considerably within populations depending

on parentage (Conover and Kynard 1981). We found no interaction between the effects of food regime and parentage on sex differentiation in zebrafish, and were surprised to find that pure-bred families were almost all male-biased or balanced while out-crossed families tended to be highly female-biased.

One potential explanation for this female bias is that it is a manifestation of Haldane's (1922) rule. If these lines are different races, and if some sort of rudimentary XX/XY system determines zebrafish sex, then the loss of males in the out-crossed crosses would be consistent with the rule. The TU and WIK lines have different origins, have been maintained separately from one another for years (www.zfin.org), and now differ at the nucleotide level to such an extent that WIK/TU crosses are routinely used to map gene locations by bulk-segregrant analysis (Nechiporuk et al. 1999).

The segregation distortion could be based on the hemizygous nature of genes on the sex chromosome of the heterogametic sex, or the release of distorter alleles in heterozygous backgrounds (Orr 2005). Uchida et al. (2002) suggested that the genetic sex of zebrafish is determined by a XX/XY-like system, based on the occurrence of highly female biased (~98%) sex ratios in the progeny from some crosses of gynogenetic diploid males (produced parthenogentially, with activation of the egg by sperm, but no fusion of sperm and egg nuclei) and wild-type females. Others have proposed a polygenic system of sex determination, based on the existence of mixed sex ratios in gynogenetically produced broods and highly variable sex ratios in broods derived from natural matings (Pelegri and Schulte-Merker 1999). However, conclusive evidence confirming or refuting either system is lacking; no genetic sex-determination system has been identified in zebrafish (Traut and Winking 2001).

Whatever the cause of strong female bias in out-crossed families, it may be adaptive. Heterozygosity contributes to developmental stability and immunological resistance to infection (e.g., Tooby 1982; Mitton 1993), and parasite resistance appears to be low in genetically monomorphic species (Reimchen and Nosil 2001). In the present study, out-crossed juveniles did not survive better or grow faster than pure-breds, but out-crossed zebrafish could be more resistant to diseases or parasites that would reduce allocation of energy to reproduction, especially in nature. If having more energy for reproduction is a benefit derived from heterozygosity, it may have a greater reproductive pay-off for females than for males, as seems to be the case for the benefits of large body size. Developmental bias toward females may thus perform the same function for both well-fed and out-crossed zebrafish: Capitalizing on high robustness to maximize reproductive fitness.

The experimental demonstration that the growth rate of a zebrafish directly influences its sexual differentiation supports our hypothesis that growth rate may underlie the influences of a number of environmental factors on sex determination in other species. That the influence of growth rate on sex determination is readily interpretable in terms of traditional sex allocation theory should encourage continued research toward identifying the adaptive explanations for other mechanisms of environmental sex determination. Growth-dependent sex differentiation may be considered an example of how developmental plasticity facilitates adaptation to variable environments. The unexpected finding that sex determination in zebrafish also depends on whether the individual is out-crossed or pure-bred may be an alternative route to differential reproductive pay-offs through male versus female function. Further study may resolve if female bias in out-crossed zebrafish can be attributed to Haldane's rule, and if this bias has adaptive significance.

Acknowledgments We thank Barry Paw for his support during these experiments. We are also grateful to Karel Liem and Hans Hoffman for helpful discussions during preliminary stages of the project, and Tracey Spoon, Carl Smith, and Les Kaufman for reviewing various versions of the paper. Finally, we also thank the three referees for their helpful criticisms of the manuscript. All experiments comply with current laws of the USA and were conducted in adherence to guidelines set forth by the institutional animal care and use committee.

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