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CORRESPONDENCE

The importance of controlling genetic variation – remarks on ‘Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response’

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Ribas et al. (2017) claim that the sex of zebrafish *Danio rerio* is influenced by stocking density. The authors describe a series of experiments in which four breeding pairs were reproduced, and the resulting larvae were stocked at four different densities and then raised to maturity. At the two higher densities, the percentage of males was significantly higher than 50%, and this was not the case at the two lower densities. Based on this evidence, it was suggested that higher stocking densities induce masculinization. However, offspring from each breeding pair were not represented in each density treatment (Fig. 1). Larvae from individual breeding pairs were split into two to 12 tanks, and tanks from breeding pairs were distributed unequally across treatments (Ribas et al., 2017, table 1). Liew et al. (2012) demonstrated that sex ratio in unselected zebrafish families can range from 5% to 97% male owing to genetic variation between individual broodstock, and therefore it is crucial that genetic variation be controlled in any study examining environmental effects on sex determination in zebrafish.

This oversight has serious consequences for the interpretation of the authors’ data. At the lowest two densities, breeding pair number one gave offspring groups with female-biased sex ratios, and breeding pair three gave male-biased offspring groups. Offspring from breeding pair one were not tested at the two higher densities, and only offspring from breeding pair three were tested at the highest density. Thus, sex ratio at the higher rearing densities was skewed towards being male-biased by genetic differences in the fish that were tested. If we examine sex ratios produced by individual breeding pairs, we see there is high variation between pairs, and it is difficult to make conclusions about any trend across treatments because pairs were not tested at all densities (Fig. 1). The appropriate experimental design would be to control for genetic variation by equally representing each breeding pair at all stocking densities.

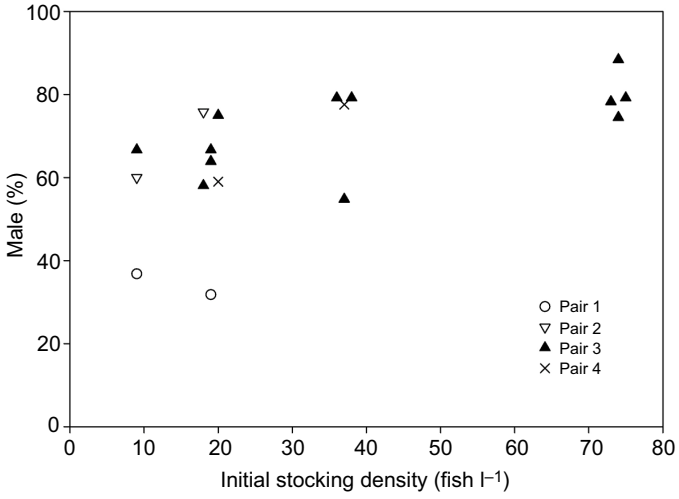

Fig. 1. Sex ratio in tanks with different initial stocking densities identified by breeding pair. Data are from table 1 of Ribas et al. (2017). Overlapping data points are offset horizontally for ease of interpretation.

Liew et al. (2012) report the results of two experiments assessing the effects of density on sex ratio in zebrafish. In the first experiment, genetic variation was not controlled – egg batches were assigned unsystematically to different density treatments – and a statistically significant difference was found, with higher densities resulting in masculinization. However, the authors then repeated the experiment and controlled for genetic variation – embryos collected on the same day were pooled, divided and assigned equally to all three density treatments – and there was no longer a statistically significant effect of density on sex ratio. This perfectly illustrates the importance of accounting for genetic variation.

Hazlerigg et al. (2012) also examined the effect of stocking density on sex ratio and did control for genetic variation by stocking larvae from the same pool of embryos in the various density treatments. That study found no relationship between the two variables. As noted by Ribas et al. (2017), this could indicate variation between the different strains (WIK and AB) used in these studies. Additionally, the maximum density examined by Ribas et al. (2017) was higher than that examined by Hazlerigg et al. (2012). However, the difference in the conclusions could also result from the more controlled experimental design used by Hazlerigg et al. (2012) that included adjusting the feeding regimen and water flow (and thereby oxygen supply and ammonia removal) with fish density to control for environmental conditions across density treatments.

Of the reported studies that examine the effect of stocking density on sex in zebrafish, those which control for genetic variation find no effect. It is possible, as has been previously proposed (Liew et al., 2012), that there is an influence of density on sex in some genetically distinct families. However, examination of this hypothesis requires further experimentation.

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Response to "The importance of controlling genetic variation – remarks on 'Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response'"

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We appreciate the comment by Delomas and Dabrowski to the Ribas et al. (2017a) paper and want to mention that we were very much aware of the possible effect of genetic differences in the sex ratio response to increasing rearing density from the beginning. This is why, in each one of the four replications of experiment 1, we avoided using pooled eggs and sperm from different progenitors, and instead used eggs and sperm derived from a single dam and sire, respectively. Thus, each time we used a different pair (pairs 1–4), hence creating four different families (biological replicates). Although some sources (e.g. FishBase) claim higher values, the absolute fecundity of zebrafish females is typically around 300 eggs (Ribas and Piferrer, 2014). This creates an experimental problem when eggs from individual dams are used, as in the Ribas et al. (2017a) paper, because with that number of available eggs it is very difficult to test four rearing densities starting with 25, 50, 100 and 200 fish per tank, as even with only two technical replicates per density the amount of eggs needed climbs to 750. Thus, as Delomas and Dabrowski rightly point out, pairs were not tested at all densities, as we explicitly show in table 1 of Ribas et al. (2017a). In that study, pairs 2, 3 and 4 showed an increase in the number of males, while pair 1 showed the opposite effect but, again, the masculinizing effect of elevated density was seen when the sex ratios obtained at each density were compared with the sex ratios of the lowest density, as also stated. Genotype-by-environment (G×E) interactions in the sex ratio response to external factors in zebrafish have been recently described by Ribas et al. (2017b), in accordance with the existence of the sex ratio variation among, but not within, families of domesticated zebrafish, as previously proposed by Liew et al. (2012). This was reflected by different susceptibilities of elevated temperature among different families (see fig. 1C of Ribas et al., 2017b).

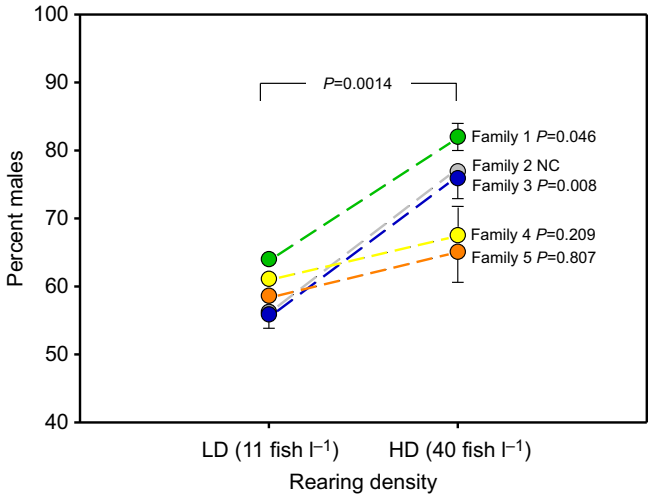

Fig. 1. Genotype-dependent sex ratio response of five different zebrafish (AB strain) families as a function of rearing density [low density (LD) =11 fish l^{-1}; high density (HD)=40 fish l^{-1}] during the sex differentiation period [18–45 days post-fertilization (dpf)]. The number of fish available for sexing at 90 dpf per family was as follows: *n*=95, 29, 201, 161 and 74 for families 1–5, respectively. Family numbers are arbitrary. Data are means±s.e.m. of two technical replicates for each family/density combination. For clarity, error bars that are similar in size to or smaller than the data points are not shown. NC, significance level not computed owing to insufficient sample size.

Furthermore, in Ribas et al. (2017a) we clearly stated that ongoing experiments in our laboratory confirm the masculinizing effects of elevated densities. We advance the sex ratio results of these experiments below, which are part of a larger study on other aspects of the effects of rearing density. We reared five additional pairs precisely to better determine the effect of genetic variation in the masculinizing response to elevated rearing density. In order to avoid the shortcomings of not having enough eggs, in this case we used only two densities: 11 and 40 fish l^{-1} in 2.7-liter tanks for the low density and high density groups, respectively, applied during the 18–45 days post-fertilization (dpf) period. Each density treatment was replicated twice for each family pair. This is possible with the typical ∼300 egg batch. As previously observed in the temperature experiments (Ribas et al., 2017b), we found a G×E interaction in the response to elevated densities as evidenced by non-parallel, family-specific reaction norms (Fig. 1). Resulting sex ratios were analyzed at 90 dpf with the chi-squared test. Of the five families tested, family 2 was excluded from the statistical analysis because of insufficient fish numbers at the time of sampling. Of the remaining four families, families 1 and 3 showed statistical differences ($P<0.05$ and $P<0.01$, respectively), whereas for families 4 and 5, although there was also an increase in the number of males, differences were not statistically significant (Fig. 1). If the data for all five families are combined, differences are significant ($P=0.0014$). These data, along with the data presented in Ribas et al. (2017a), clearly illustrate that at a rearing density of 40 fish l^{-1} or higher, masculinization occurs in zebrafish. Although most families tend to increase the number of males in response to elevated density, some show statistical significance and some do not. Thus, as stated in the concluding remarks of our initial paper, there is an inter-family variation, meaning that there is, as in many other aspects, a genetic component in the sex ratio response to rearing density.

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