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RESEARCH ARTICLE

Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response

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ABSTRACT

The zebrafish (*Danio rerio*) has become a well-established experimental model in many research fields but the loss of the primary sex-determining region during the process of domestication renders laboratory strains of zebrafish susceptible to the effects of environmental factors on sex ratios. Further, an essential husbandry aspect – the optimal rearing density to avoid stress-induced masculinization – is not known. We carried out two experiments: the first focusing on the effects of density on survival, growth and sex ratio by rearing zebrafish at different initial densities (9, 19, 37 and 74 fish per litre) for 3 months (6–90 days post-fertilization, dpf), and the second focusing on the effects of cortisol during the sex differentiation period (15–45 dpf) for zebrafish reared at low density. The results showed an increase in the number of males in groups subjected to the two highest initial rearing densities; we also observed a reduction of survival and growth in a density-dependent manner. Furthermore, zebrafish treated with cortisol during the sex differentiation period showed a complete masculinization of the population; treatment with the cortisol synthesis inhibitor metyrapone negated the effects of exogenous cortisol. Our results indicate that the process of sex differentiation in domesticated zebrafish can be perturbed by elevated stocking density and that this effect is likely to be mediated by an increase in cortisol through the stress response. However, the underlying mechanism needs further study.

KEY WORDS: Sex differentiation, Stress, Sex ratio, Cortisol, *Danio rerio*

INTRODUCTION

The zebrafish (*Danio rerio*) is a small tropical freshwater fish of the family Cyprinidae (Mayden et al., 2007) and a well-established animal model for many research fields (Streisinger et al., 1981; Chakrabarti et al., 1983; Whitfield et al., 1996; McGonnell and Fowkes, 2006; Ribas and Piferrer, 2014). Studies looking at genetic polymorphisms and using a variety of screening methods on domesticated zebrafish strains have identified putative sex-linked loci in different chromosomes: 3 and 4 (Anderson et al., 2012), 5 (Bradley et al., 2011) and 16 (Howe et al., 2013). Moreover, using different families and several crosses, family-dependent sex ratios were obtained, which led to the proposal that domesticated zebrafish have a polygenetic sex-determining system in which genetic factors and environment determine the sex (Liew et al., 2012; Ribas et al., 2017). Recently, in wild zebrafish populations it has been found that a locus at the telomeric region of chromosome 4 is strongly linked with sex and compatible with a WZ/ZZ sex determination system (Wilson et al., 2014). Interestingly, this region is not found in most laboratory strains and it has been argued that domestication has caused the loss of the sex-linked region as a result of continuous breeding and mutations (Wilson et al., 2014). Thus, because they lack the master sex-determining gene, laboratory strains of zebrafish probably have several minor sex-linked loci that determine sex, although under strong environmental influence. This would explain why laboratory zebrafish behave as though they have a polygenic sex-determining system (Liew et al., 2012). Environmental influences during early development are able to influence sex ratios in species with polygenic sex determination (Penman and Piferrer, 2008). Surprisingly, and opposite to what happens with rodents, where universal husbandry protocols are available, the zebrafish community lacks universal rearing guidelines. Although many authors have focused on the different aspects of rearing conditions (Westerfield, 1995; Casebolt et al., 1998; Trevarrow, 2004; Lawrence, 2007; Pavlidis et al., 2013; Giacomini et al., 2015), a main variable such as rearing density is still not clearly established. Thus, it is urgent to clarify the influence of rearing density on zebrafish sex differentiation.

Regardless of whether they are a laboratory model such as zebrafish or farmed species, rearing fish at a sufficiently elevated density has obvious advantages regarding space and resource utilization optimization. The influence of stocking density on fish sex ratios has only been documented in a few fish species, e.g. paradise fish, *Macropodus opercularus* (Francis, 1984), some coral reef fish species (Kuwamura et al., 2014; Lutnesky, 1994), European eel, *Anguilla anguilla* (Huertas and Cerdà, 2006; Krueger and Oliveira, 1999; Roncarati et al., 1997), European sea bass, *Dicentrarchus labrax* (Saillant et al., 2003) and zebrafish (Hazlerigg et al., 2012). However, as for any confined animal, a rearing density beyond a certain threshold has evident detrimental consequences in fish, including lower survival, decreased growth, higher incidence of deformities, increased susceptibility to diseases, and altered reproduction (e.g. Iguchi et al., 2003; North et al., 2006). The last of these is manifested in lower fecundity or higher larval mortality (Coman et al., 2007) due to an increase in plasma cortisol levels when fish are reared at elevated densities (Schreck, 1981; Barton, 2002). Cortisol is a glucocorticoid hormone regulating gluconeogenesis and other metabolic processes where glucose is needed as an energy substrate, and plays a role also in osmoregulation, growth and reproduction (Barton et al., 1987; Mommsen et al., 1999). Cortisol is considered to be a primary stress indicator with detrimental effects in fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Thus, elevated cortisol reduced survival in Atlantic cod, *Gadus morhua*, eggs (Kleppe et al., 2013) and inhibited puberty in common carp, *Cyprinus carpio* (Consten et al., 2002).

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Received 21 June 2016; Accepted 29 December 2016

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Journal of Experimental Biology (2017) 220, 1056-1064 doi:10.1242/jeb.144980

In recent years, it has become evident that elevated plasma or whole-body cortisol levels during early development can also affect the process of sex differentiation, resulting in an increase in the number of males in different species, including medaka, *Oryzias latipes* (Hayashi et al., 2010), pejerrey, *Odontesthes bonariensis* (Hattori et al., 2009), southern flounder, *Paralichthys lethostigma* (Mankiewicz et al., 2013), and Japanese flounder, *P. olivaceus* (Yamaguchi et al., 2010). However, each one of these studies proposed a distinct, albeit not necessarily mutually exclusive, mechanism on the underlying mechanism responsible for masculinization by cortisol. Furthermore, cortisol synthesis can be inhibited by metyrapone, a chemical compound that blocks the conversion of 11-deoxycortisol to cortisol (Lisansky et al., 1989). Metyrapone has been used in some fish species (e.g. zebrafish or rainbow trout, *Oncorhynchus mykiss*), as a strategy to elucidate cortisol effects (Leach and Taylor, 1980; Miranda et al., 1998; Zanuzzo and Urbinati, 2015). For example, metyrapone treatment through the diet was able to inhibit masculinization induced by high temperature in Japanese flounder (Yamaguchi et al., 2010).

Studies on the effects of rearing density in zebrafish are scarce and sometimes controversial. In a study involving eight major zebrafish facilities across the world, it was found that holding densities as high as 12 fish per litre after 4 months of age did not result in negative effects on clutch size, spawning success or egg viability (Castranova et al., 2011). When mating, changes in egg production, hatching rate or larval length were not observed until a density of 60 fish per litre (Goolish et al., 1998). Cortisol levels increased up to fourfold in adult zebrafish confined at high densities of 40 fish per litre (Ramsay et al., 2006). Differences in the number of reared zebrafish required to observe alterations in behaviour are present in the literature: 0.025 fish per litre in 38 l tanks (Larson et al., 2006), 0.25 fish per litre in 60 l tanks (Spence and Smith, 2005) and 1.4 fish per litre in 21 l tanks (Moretz et al., 2007). In contrast, other authors did not find differences at 1.2 fish per litre using 45 l tanks (Gronquist and Berges, 2013). Despite these studies, the influence of density during sexual development, when fish are more susceptible to the effects of external perturbations, is far from clear in zebrafish. One study found that elevated density caused a decrease in growth and survival rates without a clear link with sex ratios (Hazlerigg et al., 2012). Another study found that high density increased the number of males, although there was a high inter-family variation in the response, suggesting that other factors, both genetic and environmental, could also be affecting sex ratios (Liew et al., 2012).

In this study, we took advantage of the fact that domesticated zebrafish are sensitive to environmental perturbations to address the general question of how environmental factors can influence the process of sex differentiation in fish and, in particular, how stocking density affects the sex ratio. In addition, and in order to study the possible role of cortisol in the masculinization of zebrafish subjected to stress confinement as a result of high density, synthetic cortisol and metyrapone were administered during the sex differentiation period.

MATERIALS AND METHODS

Animal rearing conditions

Domesticated zebrafish (AB strain) were housed in a commercial rack (Aquaneering, San Diego, CA, USA) fitted with a recirculating water system (supplied with a water pump of 6000 l h^{-1}) and placed in an *ad hoc* chamber facility in our institute subjected to a constant photoperiod (12 h light:12 h dark), air temperature of 26±1°C and humidity of 50±3%. Water quality parameters were monitored daily and included: temperature 28±0.2°C, pH 7.2±0.5, conductivity 750–900 µS and dissolved oxygen 6.5–7.0 mg l^{-1}. Other water quality parameters were checked periodically (2–3 times a month) by the Water Analysis Service of our institute and maintained in the appropriate ranges (Ribas and Piferrer, 2014): ammonium 0.03±0.00 mg l^{-1}, nitrite 0.25±0.14 mg l^{-1}, nitrate 66.42±8.04 mg l^{-1}, silicate 15.22±2.53 mg l^{-1} and phosphate 32.34±7.43 mg l^{-1}.

Breeding was always performed by natural spawning after single-pair crossings. The total number of eggs and fertilized embryos was counted to ensure that fecundity was according to the reference values for this species (Ribas and Piferrer, 2014) and post-hatch viability in accordance with the OECD's guidelines for the Fish Sexual Development Test (OECD, 2011; Fig S1). Eggs were reared in Petri dishes (ThermoFisher Scientific, Waltham, MA, USA) at ∼50 eggs per dish filled with embryo medium (pH 7.2±0.5), supplemented with 0.1% Methylene Blue (Sigma-Aldrich, Madrid, Spain) at 26±1°C until 6 dpf. Then, hatched larvae were transferred to tanks at 6 dpf and housed in the commercial rack described above.

Fish were fed *ad libitum* 3 times a day with a commercial food according to their developmental stages: 6–15 days post-fertilization (dpf) larvae were fed with Micron (Sera, Heinsberg, Germany), which contains natural plankton (50% spirulina and 16% krill); 15–40, 40–60 and 60–90 dpf fish were fed with pellets of increasing size: ST1, ST2 and ST3, respectively (AquaSchwarz, Göttingen, Germany), containing 54–59% crude protein, 15–16% lipids, 12% crude ash, vitamins A, D3, E, C (C at 100–2000 mg kg^{-1}) and omega-3 (HUFA at 28–30 mg g^{-1}). The commercial feed in all stages was supplemented with live *Artemia* nauplii (AF48, INVE Aquaculture, Dendermonde, Belgium). The debris at the bottom and walls of the tanks was cleaned 3 times a week. Fish were kept in accordance with the approved institutional guidelines on the use of animals for research purposes and in agreement with the European regulations of animal welfare (ETS N8 123, 01/01/91).

Experiment 1: effects of stocking density

Fish were reared in tanks (Aquaneering, model ZT280) of a nominal volume of 2.8 l (the actual capacity was 2.7 l). Different densities were achieved by placing 6-dpf larvae in the following numbers in the 2.7 l actual available volume: 25, 50, 100 and 200 larvae. This gave initial densities of 9.25, 18.51, 37.03 and 74.07 fish per litre. For clarity purposes, the rounded values of 9, 19, 37 and 74 fish per litre will be used from now on. The experiment was repeated 4 times with four different families, and each density treatment was replicated between 3 and 7 times, depending on the fecundity of each family. In total, 1625 fish were used. Survival for each density treatment was recorded at 15, 20, 30, 50, 70 and 90 dpf. At 50 dpf, juvenile fish from the 9 and 74 fish per litre groups were euthanized by immersion into ice for cortisol analysis (see below for further details). At 90 dpf, fish were euthanized on iced water followed by decapitation and total body mass (M_b, precision ±0.05 g), standard length (SL, precision ±0.01 cm) and sex ratios were recorded. The Fulton's condition factor (k) was calculated following the formula: $k=(M_b\times100)/SL^3$ (Fulton, 1902).

Experiment 2: cortisol treatment

The spawn of three different pairs were pooled in each experimental tank (with a total of 36 fish per 2.8 l tank), and received one of the following randomly assigned treatments: control (C), cortisol (F; hydrocortisone; ref. H0888, Sigma-Aldrich), metyrapone (M; 2-methyl-1,2-di-3-pyridyl-1-propanone; ref. 856525, Sigma-Aldrich), cortisol plus metyrapone (F+M), and the synthetic

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androgen 17α-methyltestosterone (MT; ref. M7252, Sigma-Aldrich) as a positive control for masculinization. Treated feed was prepared with the following concentrations (µg g^{-1} feed). F group: 50; F+M group: 50+500; M group: 500; MT group: 50. All compounds were diluted with 3 ml of 96% ethanol and sprayed directly on feed. Feed in the control group was also sprayed with 96% ethanol. The treated feed was then air dried in a ventilated hood for 3 h to remove ethanol traces and then stored at −20°C until consumption. For each group, at least two replicates were used, with a total number of 444 fish in the whole experiment. Fish survival calculations and sampling procedures were performed as described above for experiment 1.

Coinciding with the sex differentiation period (15–45 dpf), tanks were removed from the recirculating water system housing rack and placed in groups of three inside a large tub filled with water, maintained at a constant water temperature of 27.63±0.11°C with the use of electric waterproof heaters. Tanks were individually oxygenated with an independent air flow source but without altering environmental conditions and natural swimming. Renewal of three-quarters of the tank water was performed 3 times a week, together with removal of the debris at the bottom of the tanks. During this period, fish were fed 3 times a day with the treated feed. Afterwards (at 45 dpf), tanks were moved back to the housing rack.

Whole-body cortisol measurement

Whole-body cortisol levels were measured in juvenile 50 dpf zebrafish samples using a commercial enzyme immunoassay kit (ref. 402710, Neogen, Lansing, MI, USA), following the manufacturer's instructions with slight modifications. The specificity of the test was evaluated by comparing the samples with a standard curve. The linear regression of the standard curve was R^2=0.979. The mean intra-assay coefficient of variation for all tests was always <10% with an assay sensitivity of 0.03 ng ml^{-1}. Frozen fish were homogenized individually in 100 µl cold phosphate-buffered saline (PBS, pH 7.4) using a glass pestle, then suspended in 2 ml of pre-cooled diethyl ether for 15 min at 4°C. Homogenates were centrifuged at 2500 ***g*** for 2 min and frozen at −80°C for 30 min to recover the organic phase. This step was repeated 3 times. The diethyl ether from each sample was evaporated using a dry heater at 30°C in a ventilated hood. Samples were immediately resuspended in extraction buffer supplied by the manufacturer and diluted 1:5. Tubes containing samples and standards were measured at 650 nm in a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). All samples were measured in duplicate. Cortisol levels of a total of 23 juvenile fish were measured: eight fish in the low-density group (from the 9 fish per litre group) and 15 fish in the high-density group (from the 74 fish per litre group). The mean M_b of the fish at each density was used to calculate whole-body cortisol per gram of fish.

Statistical data analysis

Data normality and the homoscedasticity of variances were checked with Kolmogorov–Smirnov's and Levene's tests, respectively. When data did not follow normality, a Box Cox transformation was applied. One-way analysis of variance (ANOVA) was used to detect possible differences among groups in survival, M_b, SL and k calculations. *Post hoc* multiple comparisons were carried out with Tukey's test. For male SL and male k data, the Kruskal–Wallis test was used. Student's t-test was used to detect differences in cortisol levels. The Chi-squared test with Yate's correction was used for sex ratio analysis (Fowler et al., 2008). All data analyses were performed with Stats Graphics software (version 17). Data are expressed as means±s.e.m. In all tests, differences were accepted as significant when P<0.05.

RESULTS

Experiment 1

Effect of rearing density on survival

Survival was inversely related to rearing density (Fig. 1). Fish survival at 90 dpf was 76%, 64%, 45% and 35% relative to the initial number of fish in each case for the 9, 19, 37 and 74 fish per litre groups, respectively. The two highest tested densities significantly decreased (P<0.05) survival when compared with the two lowest densities at all sampling points (Fig. 1A). The highest mortality was observed between 6 and 15 dpf but survival was density independent after this point (Fig. 1B).

Effect of rearing density on growth

Growth was also inversely related to rearing density, with sex-related differences (Fig. 2). M_b was significantly decreased (P<0.05 and P<0.01) in males for groups reared at a density of 19 fish per litre or higher, but in females such an effect was observed only with densities of 37 fish per litre or higher (P<0.05) (Fig. 2A). SL was

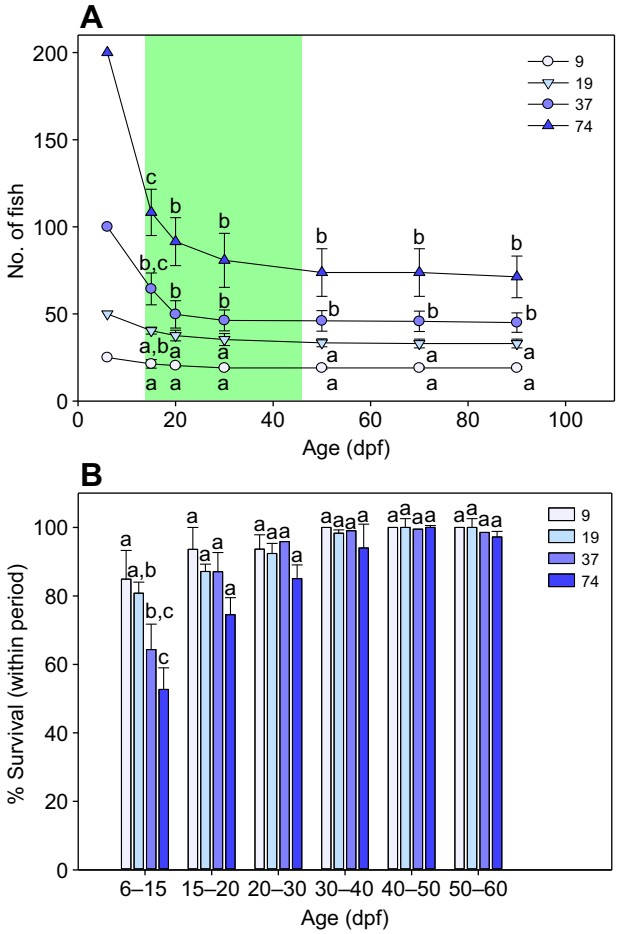

Fig. 1. Zebrafish survival as a function of stocking density during the first 3 months of age. Fish were held at densities of 9, 19, 37 and 74 fish per litre. (A) Absolute survival from 6 to 90 days post-fertilization (dpf). The sex differentiation period (15–45 dpf) is shaded in green. (B) Survival relative to different age periods. Data are presented as means±s.e.m. of 3–6 biological replicates per group. Significant differences (P<0.05) among groups at a given sampling age period were tested by one-way ANOVA and are indicated by different letters.

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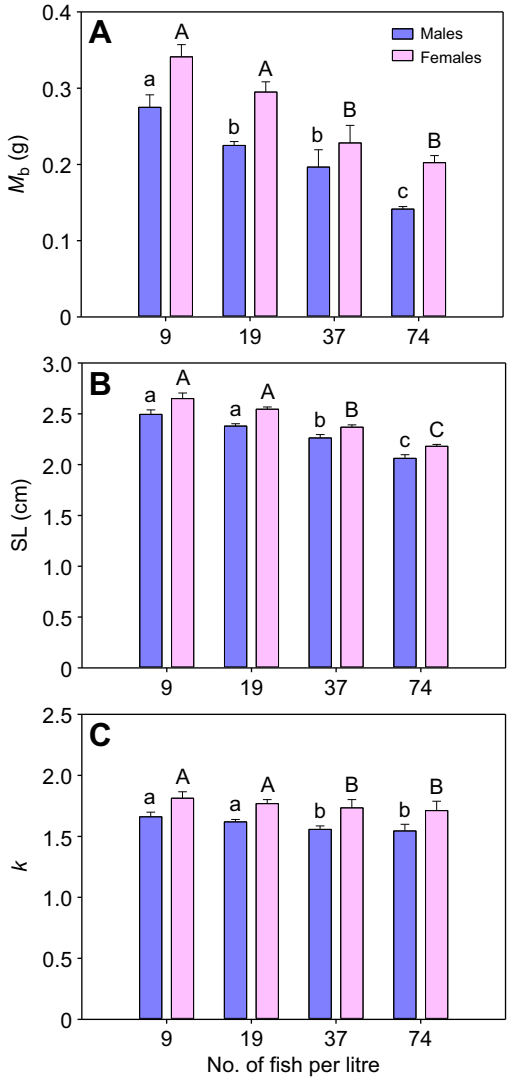

Fig. 2. Zebrafish growth as a function of different stocking density during the first 3 months of age and sex. (A) Body mass (M_b), (B) standard length (SL) and (C) condition factor (k). Data are presented as means±s.e.m. of 3–6 biological replicates per group. The number of fish at 90 dpf was 57, 231, 181 and 280 for the 9, 19, 37 and 74 fish per litre groups, respectively. Within the same sex, different letters indicate significant differences (a–b or A–B, P<0.05; and a–c or A–C, P<0.01) between groups analysed by ANOVA, except for SL and k male data, which were analysed by a Kruskal–Wallis test.

significantly (P<0.05) decreased in both sexes at densities of 37 fish per litre and higher (Fig. 2B). A similar trend of density effects was observed for k (Fig. 2C).

Effect of rearing density on sex ratio

There was a density-dependent effect on zebrafish sex ratio as the number of males observed at 90 dpf increased with rearing density (Fig. 3A). At a density of 9 and 19 fish per litre, the number of males was 54.4±11.06% and 61.4±6.04%, respectively, a proportion not different from the expected Fisherian sex ratio. Significant differences with respect to the 9 fish per litre group were recorded with densities of 37 fish per litre (71.6±6.5%; P<0.05) and 74 fish per litre (80.1±3.4%; P<0.01).

In order to determine density effects when sex differentiation takes place (15–45 dpf), the number of fish alive in the tank during this process was calculated by averaging observed values at 15, 20, 30 and

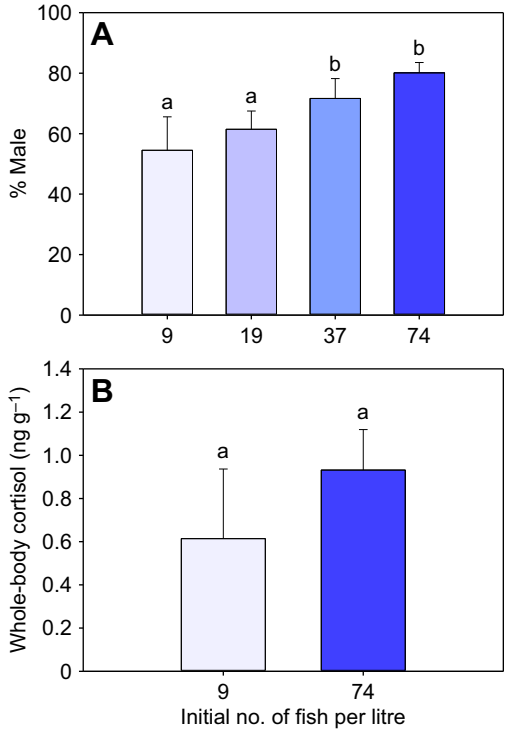

Fig. 3. Sex ratio and whole-body cortisol levels in zebrafish reared at different densities. (A) Zebrafish sex ratio as a function of stocking density during the first 3 months of age. Data are presented as means±s.e.m. of 3–6 biological replicates per group. The final number of fish per group is as in Fig. 2. Significant differences among groups (indicated by different letters) were analysed by a Chi-squared test with Yate's correction (P<0.05 in 37 fish per litre group and P<0.01 in 74 fish per litre group) with respect to the group with the lowest rearing density. (B) Whole-body cortisol levels in juvenile zebrafish at 50 dpf as a function of stocking density. Data are presented as means±s.e.m. of 8 (9 fish per litre) or 15 biological replicates (74 fish per litre). There was no significant difference between groups (Student's t-test, P=0.49).

50 dpf for each replicate treatment (Table 1). The mean±s.e.m. number of fish per litre was 7.4±0.4, 13.5±0.3, 18.3±0.7 and 31.9±1.0 in the 9, 19, 37 and 74 fish per litre groups, respectively.

Effect of rearing density on whole-body cortisol levels

Whole-body cortisol levels in fish subjected to high density confinement over 45 days (from 6 dpf to 50 dpf) increased at the end of this period by ~50% in the 74 versus the 9 fish per litre groups (Fig. 3B). However, these differences were not significant (P=0.49).

Experiment 2

Effect of cortisol on survival

At the end of the experiment (90 dpf), survival of the control group in experiment 2 was lower than that of the control group in experiment 1. Treatment with M or MT did not affect survival. However, treatment with F, particularly when it was administered alone, significantly (P<0.05) increased survival (Fig. 4).

Effect of cortisol on growth

Male M_b in the F, F+M and MT groups was significantly reduced (P<0.05) when compared with the control group but not with the M group (Fig. 5A). Treated females did not show any statistical differences in M_b or SL (the MT group contained only two females; Fig. 5A,B). Males treated with synthetic cortisol showed a significant decrease in SL (P<0.05; Fig. 5B). No significant differences were found in k in either males or females when compared with control fish (Fig. 5C).

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Table 1. Stocking density effects on zebrafish survival during the period of sex differentiation and on adult sex ratio

		Initial		Sex differentiation period			Final		
Pair	No. of replicates	No. of fish	No. of fish per litre	No. of fish per tank	No. of fish per litre	No. of fish per litre per group	No. of fish per tank	% Males	*P*-value
1	3	25	9	19.0±0.0	7.0	7.4±0.4	19	36.8	
2			9	20.5±0.4	7.6		20	60	
3			9	20.5±0.7	7.6		18	66.7	ns
1	7	50	19	27.3±0.5	10.1	13.5±0.3	22	31.8	
2			19	36.8±0.6	13.6		33	75.8	
3			19	37.8±0.0	14.0		36	63.9	
3			19	46.7±1.2	17.3		40	75	
3			19	31.3±0.8	11.6		30	66.7	
3			19	34.3±0.9	12.7		31	58.1	
4			19	42.0±0.7	15.6		39	59	ns
3	4	100	37	31.0±0.0	11.5	18.3±0.7	31	54.8	
3			37	49.3±0.0	18.2		48	79.2	
3			37	60.0±0.7	22.2		53	79.2	
4			37	57.8±1.3	21.4		49	77.6	<0.05
3	4	200	74	121.5±1.6	45.0	31.9±1.0	98	74.5	
3			74	74.0±1.7	26.3		69	88.4	
3			74	78.5±1.0	29.1		60	78.3	
3			74	71.0±1.7	24.9		53	79.2	<0.01

Initial number of fish per litre has been rounded to the nearest whole number. The sex differentiation period was from 15 to 45 days post-fertilization (dpf); data are means±s.e.m. Final values were measured at 90 dpf. *P*-value indicates departure from Fisherian sex ratio; ns, not significant.

Effect of cortisol on sex ratio

Treatment with cortisol resulted in a complete masculinization of the population ($P< 0.001$; Fig. 6). The same effect occurred with the positive control treatment MT, where 92.3±6.7% of the fish were masculinized ($P<0.001$). No differences in sex ratios were found in the M and M+F groups (61.9±0.6% and 48.6±2.7% of males, respectively) with respect to the control group (44.1±3.2%), meaning that metyrapone counteracted cortisol effects, preventing masculinization.

DISCUSSION

In this study, we tested four stocking densities for their effect on survival, growth and sex ratio of domesticated zebrafish. Consistent with the results of Hazlerigg et al., (2012), stocking zebrafish in high densities was detrimental to their survival, as also observed in other fish species including the Nile tilapia, *Oreochromis niloticus* (Huang and Chiu, 1997), pufferfish, *Takifugu rubripes* (Kotani et al., 2009), vundu catfish, *Heterobranchus longifilis* (Coulibaly et al., 2007), and pigfish, *Orthopristis chrysoptera* (DiMaggio et al., 2014).

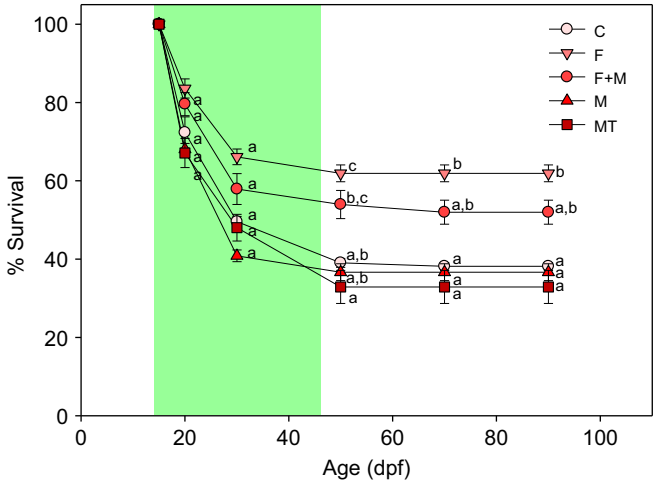

Fig. 4. Zebrafish survival during the first 3 months of age as a function of cortisol treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. The final number of fish was 34, 75, 21, 37 and 26 fish per group, respectively. The sex differentiation period (15–45 dpf) is shaded in green. Data are presented as means±s.e.m. of 2–3 biological replicates per group. Significant differences ($P<0.05$) among groups at a given sampling age were examined by one-way ANOVA and are indicated by different letters.

The stocking densities used in this study were set up at the time of transfer of the larvae to the rearing tanks but no attempt was made to replace dead fish with new ones to maintain the initial number of fish during the experiment. Thus, effects on growth and sex ratios determined at 90 dpf have to be seen as the result of the cumulative effects of varying stocking density as some fish died while others grew. Initial rearing densities of 9 and 19 fish per litre did not have any effect on sex ratios at 90 dpf. In contrast, initial densities of 37 and 74 fish per litre significantly increased the proportion of males. It should be noted that rearing fish at 9 and 19 fish per litre did not affect survival during 6–15 dpf, or SL and condition factor *k*, presumably indicating no influence of conditions other than rearing density, although a density of 19 fish per litre decreased M_b. Furthermore, survival of the group reared at 9 fish per litre was around 76%. This value is standard for zebrafish and suggests that rearing conditions other than density were not deleterious. Thus, it is probably safe to state that, based on our results, a stocking density in the range of 13–20 fish per litre in a ∼3 l commercial tank starting at 6 dpf would not cause masculinization. However, with improvements in feeding or diet formulation (e.g. supplemented by rotifers), survival in the initial stages could be higher than the survival recorded in this study. Thus, based on our data, in order to avoid density-induced masculinization, the stocking density should be taken into account, especially during the sex differentiation period. Ongoing experiments in our lab involving additional families show that elevated rearing density during this period results in a clear sex bias towards males, confirming the present results. Following the above-mentioned guidelines, in recent experiments in our lab, we did not observe masculinization. We do not know for sure whether, taking into account other factors (e.g. social interactions, behaviour, husbandry strategies, etc.) applied in

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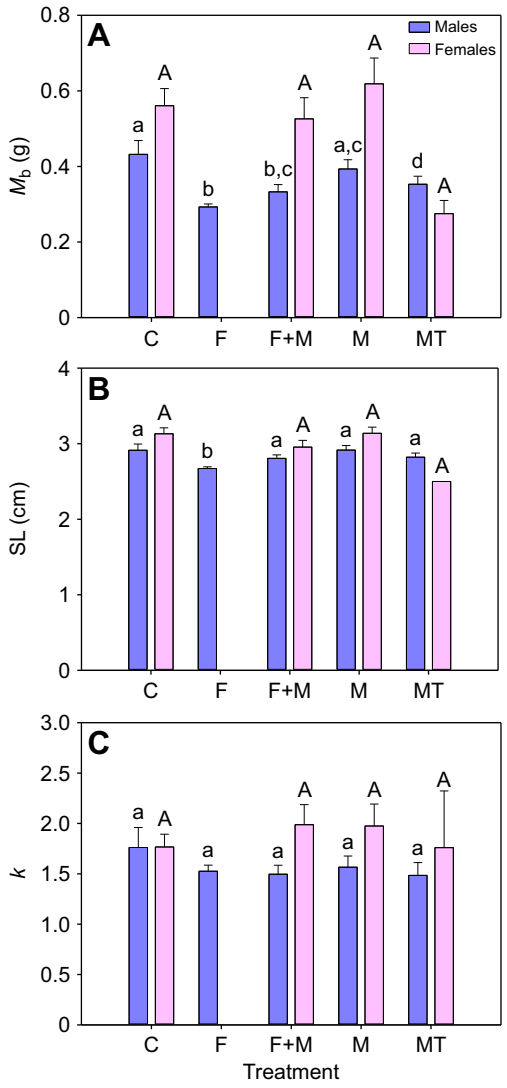

Fig. 5. Zebrafish growth during the first 3 months of age as a function of cortisol treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. (A) M_b, (B) SL and (C) *k*. Data are presented as means±s.e.m. of 2–3 biological replicates per group. The final number of fish per group is as in Fig. 4. Within the same sex, different letters indicate significant differences (P<0.05) between groups analysed by ANOVA, except for SL and *k* male data, which were analysed by a Kruskal–Wallis test.

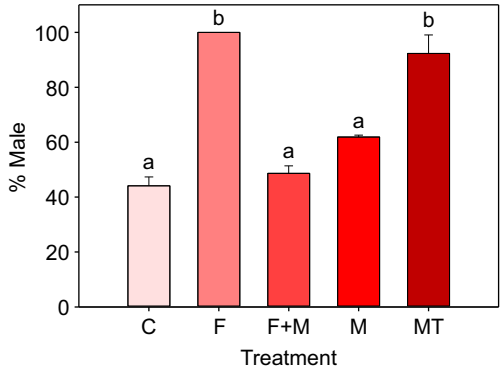

Fig. 6. Zebrafish sex ratio at 3 months of age as a function of treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. Data are presented as means±s.e.m. of 2–3 biological replicates per group. The final number of fish per group is as in Fig. 4. Significant differences among groups were analysed by the Chi-squared test with Yate's correction. Different letters indicate a significant difference (P<0.001) with respect to the control group.

other laboratories, this density range would also work well with tanks of much larger volume, e.g. 45 l tanks, as used by Gronquist and Berges (2013) for behavioural studies. In our experiments with domesticated zebrafish, other than density, environmental conditions (e.g. water quality, feeding regime, etc.) were the same in all tanks. Thus, although the sex ratio of domesticated zebrafish may be influenced by several factors, in this study we focused on rearing density, and aimed to minimize other possible environmental influences.

An interesting aspect is whether the results obtained in this study would apply to other zebrafish strains. We used domesticated zebrafish from the AB strain, but other laboratories have carried out density experiments using other zebrafish strains. Liew et al. (2012), using a Tübingen (TU) strain, found an increase in males of ∼20% with rearing densities of 66.6 fish per litre when compared with 33.3 and 16.6 fish per litre. In contrast, Hazlerigg et al. (2012), using the WIK (Wild Indian Karyotype) strain, found that densities from 2 to 40 fish per litre had no effect on sex ratios when combined with different feeding regimes (constant or limited). Here, it is interesting to note that while the WIK strain has a WZ/ZZ sex determination system with a putative sex-determining gene at the tip of chromosome 4, the TU and AB strains have lost this gene, presumably during the many manipulations in the process of domestication (Wilson et al., 2014). Because of this loss, the TU and AB strain sexual development is more sensitive to environmental cues. This concurs with the fact that domesticated zebrafish show a wide range of inter-family variation in sex ratios and that a polygenic system of sex determination has been proposed for domesticated zebrafish (Liew et al., 2012; Ribas et al., 2017; see Table 1). Taken together, it can be concluded that the effects of density found in this study with the AB strain would also apply to the TU strain, while experiments with the WIK strain are needed in order to determine whether the presence of the WZ/ZZ system confers greater resistance to the influence of environmental cues on sex ratios.

We also found that growth is inversely related to stocking density in zebrafish, as also found by Hazlerigg et al., (2012) and conforming to what has been observed in other species (Barton et al., 1987; Björnsson, 1994; Holm et al., 1990). The fish reared at the two lowest densities in this study had a mean M_b of ∼0.25±0.01 and ∼0.32±0.01 g and a mean fork length of ∼2.4±0.04 and ∼2.6±0.05 cm at 90 dpf for males and females, respectively, and corresponding with the range for the typical M_b and length of an adult zebrafish at this age (reviewed in Ribas and Piferrer, 2014). However, the highest stocking density tested produced a decrease in M_b, with mean values of ∼0.14±0.003 and ∼0.20±0.003 g for M_b and ∼2.1±0.03 and ∼2.2±0.01 cm for SL in males and females, respectively. Similar results were found in other experiments stocking zebrafish at a density of 60 fish per litre with a constant feeding regime, in which a decrease to ∼2.0 cm in length was also observed (Hazlerigg et al., 2012). In addition, in experiment 2, males treated with different compounds showed significant differences in growth rate, whereas in females those differences were not present. Specifically, M_b was reduced in fish from groups treated with cortisol, in agreement with what has previously been described for other fish species, e.g. goldfish (Bernier et al., 2004), largemouth bass, *Micropterus salmoides* (O'Connor et al., 2011),

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and sturgeon, *Huso huso* (Poursaeid et al., 2012), where cortisol treatment induced a decrease in food intake and hence a decrease in growth rate in a dose-dependent manner.

It has been observed that fast growth during the period of gonad formation promotes female development in zebrafish, suggesting that the growth rate during this period could be regarded as an environmental factor capable of affecting sex differentiation (Lawrence et al., 2008). In our experiments, fish were fed *ad libitum*, so although food intake was not strictly controlled, food was not a limiting factor for any of the groups. This reinforces the view that stocking density rather than possible differences in growth is responsible for the observed changes in sex ratio in the groups exposed to the highest stocking density.

High stocking density may be experienced as a stressful situation by the fish and if so it is then possible that the observed masculinization is somehow related to this stress response. As cortisol is the most common indicator of the stress response, to address this we measured whole-body cortisol levels in the fish reared at the lowest versus the highest stocking density tested in this study. Although whole-body cortisol levels were ∼50% higher in the latter group, differences were not significant because of the large inter-individual variability within each group. However, the most important aspect to consider is that the cortisol levels measured in our fish (<1 ng g^{-1} M_b) cannot be considered representative of a stressful situation, as reported values in zebrafish subjected to different types of stress (e.g. crowding, handling, visual predator) are much higher, in the range 4–12 ng g^{-1} (Ramsay et al., 2006; Barcellos et al., 2007; Pavlidis et al., 2013). The lack of a cortisol-related stress response has been documented in other species such as the gilthead sea bream, *Sparus aurata* (Barton et al., 2005), and the wedge sole, *Dicologoglossa cuneate* (Herrera et al., 2015), suggesting that acclimation to a chronic stressor allowed the attenuation of cortisol release, which is more related to the acute rather than the chronic stress response. This, of course, does not preclude that elevated stocking density caused elevated cortisol levels during the sensitive period of sex differentiation. Recently, it has been shown that feeding zebrafish with cortisol over a period of 5 days did not change whole-body cortisol levels but increased ovarian cortisol levels twofold (Faught et al., 2016).

Thus, in order to further explore the possible link between the stress response and masculinization, we conducted experiment 2, focusing on the possible role of cortisol in sex differentiation. Treatment with cortisol resulted in 100% masculinization, demonstrating that cortisol was able to strongly influence the process of sex differentiation in zebrafish. Similar effects were observed in other fish species, i.e. medaka (Hayashi et al., 2010), Japanese flounder (Yamaguchi et al., 2010) and pejerrey (Hattori et al., 2009) – in the latter, fish showed elevated cortisol levels after being exposed to high temperature. In the Southern flounder, the stress caused by the background colour of the tank increased cortisol levels and skewed sex ratios towards males (Mankiewicz et al., 2013).

Cortisol may also influence survival. In experiment 2, survival of the control group was lower than in experiment 1 and this is probably due to the fact that in experiment 2 all groups, including the control group, were removed from the main rack to administer the different treatments. However, the two groups treated with cortisol showed higher survival rates when compared with the rest of the groups. This runs counter to what is described in the literature in the sense that elevated cortisol plasma levels are detrimental to fish survival, and we have no satisfactory explanation for this observation. However, it has been shown that cortisol also possesses some benefits because in fish it can activate the innate immune system to better cope with adverse situations (MacKenzie et al., 2006). Thus, the increase in survival after cortisol treatment, which is clear based on our results, may be linked to these supposed benefits of cortisol. However, further work needs to be done to provide more evidence.

Treatment with metyrapone alone, a cortisol synthesis inhibitor (Leach and Taylor, 1980), had no effect on zebrafish sex ratios but completely suppressed cortisol-induced masculinization. This is interesting because in Japanese flounder treated with equal doses of the two compounds, cortisol was able to override the inhibitory effects of metyrapone on cortisol synthesis and, consequently, complete masculinization was observed (Yamaguchi et al., 2010). The metyrapone dose used in our study was 10 times higher than the cortisol dose, following the principle of a 1:10 ratio of stimulator to inhibitor in pharmacology. Thus, it may be that the effect of metyrapone as a blocker of endogenous cortisol synthesis was stronger than the effect of exogenous cortisol.

Our study demonstrates a link between high stocking density and masculinization, probably mediated by cortisol through the stress response. However, the underlying molecular mechanism is not known and thus the question of how cortisol interacts with gonadal differentiation remains unclear. To date, different – although not necessarily mutually exclusive – molecular mechanisms by which cortisol could masculinize fish gonads have been proposed. In medaka, it has been suggested that cortisol suppresses germ cell proliferation, which is associated with female development in teleosts, through inhibition of expression of the follicle-stimulating hormone receptor (*fshr*) gene (Hayashi et al., 2010). In the pejerrey, cortisol interacts with the glucocorticoid response element in the promoter of the 11β-hydroxysteroid dehydrogenase type 2 (*hsd11β2*) gene, a key enzyme that is shared between the glucocorticoid and androgen pathways, increasing its expression. This led Fernandino et al., (2012, 2013) to suggest that while increased *hsd11β2* expression contributes to the degradation of cortisol to cortisone, it also helps to convert 11β-hydroxyandrogens into 11-ketotestosreone, the typical teleost androgen, with more masculinizing potency than testosterone (Piferrer et al., 1993). In contrast, in the Japanese flounder, it was suggested that cortisol suppressed gonadal aromatase (*cyp19a1a*) expression by blocking the cAMP response element located in the *cyp19a1a* promoter (Yamaguchi et al., 2010). Thus, a clear and unifying view of the molecular mechanisms responsible for cortisol-induced masculinization is not available in fish and further research is needed.

In conclusion, we have shown that the rearing of domesticated zebrafish – which lack the master sex-determining gene present in their wild counterparts, and hence behave as though they possess a polyfactorial system of sex determination – is very susceptible to environmental perturbations in terms of sex ratio. Thus, stocking domesticated zebrafish larvae at high densities has an impact on the subsequent adult sex ratio by increasing the number of males, but also by decreasing growth and fish survival in an inversely dependent manner. We suggest that masculinization by high density may be related to the stress response and that cortisol may have a prominent role, but the underlying mechanism needs further elucidation. Although inter-family variation in the final sex ratio is a factor that cannot be underestimated in domesticated zebrafish, we suggest that when transferring larvae to the tanks, typically at 6 dpf, the initial stocking density should not be higher than 13–20 fish per litre. In any case, and with the information available now, during the process of sex differentiation (15–45 dpf), it is advisable that the lower figure of this range is not surpassed. This will avoid

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detrimental effects on survival and growth, and will prevent male-biased sex ratios in the subsequent adult population. In addition to providing new information on environmental effects on fish sex ratios, our study thus offers useful information on how to rear zebrafish, filling a gap in an essential husbandry aspect in this important experimental model.

Acknowledgements
We thank Laura Oller and David Rufete for their help with fish husbandry.

Competing interests
The authors declare no competing or financial interests.

Author contributions
F.P. and L.R. designed the study. L.R., A.V. and N.D. conducted the experiments and the subsequent analysis. L.R. drafted the initial manuscript. L.R., A.V., N.D. and F.P. wrote the manuscript. All authors read and approved the final manuscript.

Funding
This study was supported by Ministerio de Economía y Competitividad grants from Spanish government ('EpiFarm': AGL2013-41047-R) to F.P. L.R. was supported by an Epifarm contract; A.V. and N.D. were supported by Ministerio de Economía y Competitividad PhD scholarships (BES-2014-069051 and BES-2007-14273, respectively).

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.144980.supplemental

References

Anderson, J. L., Rodríguez Marí, A., Braasch, I., Amores, A., Hohenlohe, P., Batzel, P. and Postlethwait, J. H. (2012). Multiple sex-associated regions and a putative sex chromosome in zebrafish revealed by RAD mapping and population genomics. *PLoS ONE* **7**, e40701.

Barcellos, L. J. G., Ritter, F., Kreutz, L. C., Quevedo, R. M., da Silva, L. B., Bedin, A. C., Finco, J. and Cericato, L. (2007). Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, Danio rerio. *Aquaculture* **272**, 774-778.

Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.* **42**, 517-525.

Barton, B. A. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Ann. Rev. Fish Dis.* **1**, 3-26.

Barton, B. A., Schreck, C. B. and Barton, L. D. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Dis. Aquat. Organ.* **2**, 173-185.

Barton, B. A., Ribas, L., Acerete, L. and Tort, L. (2005). Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling. *Aquacult. Res.* **36**, 172-179.

Bernier, N. J., Bedard, N. and Peter, R. E. (2004). Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. *Gen. Comp. Endocrinol.* **135**, 230-240.

Björnsson, B. (1994). Effects of stocking density on growth-rate of halibut (*Hippoglossus hippoglossus* L.) reared in large circular tanks for three years. *Aquaculture* **123**, 259-270.

Bradley, K. M., Breyer, J. P., Melville, D. B., Broman, K. W., Knapik, E. W. and Smith, J. R. (2011). An SNP-based linkage map for zebrafish reveals sex determination loci. *G3 (Bethesda)* **1**, 3-9.

Casebolt, D. B., Speare, D. J. and Horney, B. S. (1998). Care and use of fish as laboratory animals: current state of knowledge. *Lab. Anim. Sci.* **48**, 124-136.

Castranova, D., Lawton, A., Lawrence, C., Baumann, D. P., Best, J., Coscolla, J., Doherty, A., Ramos, J., Hakkesteeg, J., Wang, C. et al. (2011). The effect of stocking densities on reproductive performance in laboratory zebrafish (*Danio rerio*). *Zebrafish* **8**, 141-146.

Chakrabarti, S., Streisinger, G., Singer, F. and Walker, C. (1983). Frequency of gamma-ray induced specific locus and recessive lethal mutations in mature germ cells of the zebrafish, *Brachydanio rerio*. *Genetics* **103**, 109-123.

Coman, G. J., Arnold, S. J., Jones, M. J. and Preston, N. P. (2007). Effect of rearing density on growth, survival and reproductive performance of domesticated *Penaeus monodon*. *Aquaculture* **264**, 175-183.

Consten, D., Keuning, E. D., Bogerd, J., Zandbergen, M. A., Lambert, J. G. D., Komen, J. and Goos, H. J. Th.(2002). Sex steroids and their involvement in the cortisol-induced inhibition of pubertal development in male common carp, *Cyprinus carpio* L. *Biol. Reprod.* **67**, 465-472.

Coulibaly, A., Oulattara, I. N., Koné, T., N'Douba, V., Snoeks, J., Gooré Bi, G. and Kouamélan, E. P. (2007). First results of floating cage culture of the African catfish *Heterobranchus longifilis* Valenciennes, 1840: effect of stocking density on survival and growth rates. *Aquaculture* **263**, 61-67.

DiMaggio, M. A., Ohs, C. L., Broach, J. S. and Sink, T. D. (2014). Effects of stocking density on growth, survival, and stress physiology of pigfish. *N. Am. J. Aquacult.* **76**, 201-210.

Faught, E., Best, C. and Vijayan, M. M. (2016). Maternal stress-associated cortisol stimulation may protect embryos from cortisol excess in zebrafish. *R. Soc. Open Sci.* **3**, 160032.

Fernandino, J. I., Hattori, R. S., Kishii, A., Strüssmann, C. A. and Somoza, G. M. (2012). The cortisol and androgen pathways cross talk in high temperature-induced masculinization: the 11 beta-hydroxysteroid dehydrogenase as a key enzyme. *Endocrinology* **153**, 6003-6011.

Fernandino, J. I., Hattori, R. S., Moreno Acosta, O. D., Strüessmann, C. A. and Somoza, G. M. (2013). Environmental stress-induced testis differentiation: Androgen as a by-product of cortisol inactivation. *Gen. Comp. Endocrinol.* **192**, 36-44.

Fowler, J., Cohen, L. and Jarvis, P. (2008). *Practical Statistics for Field Biology*, 2nd edn. Chichester, West Sussex: John Wiley & Sons.

Francis, R. C. (1984). The effects of bidirectional selection for social-dominance on agonistic behavior and sex-ratios in the paradise fish (*Macropodus opercuularis*). *Behaviour* **90**, 25-45.

Fulton, T. (1902). Rate of growth of seas fishes. *Sci. Invest. Fish. Div. Scot* **20**, 326-439.

Giacomini, A. C. V. V., de Abreu, M. S., Koakoski, G., Idalêncio, R., Kalichak, F., Oliveira, T. A., da Rosa, J. G. S., Gusso, D., Piato, A. L. and Barcellos, L. J. G. (2015). My stress, our stress: Blunted cortisol response to stress in isolated housed zebrafish. *Physiol. Behav.* **139**, 182-187.

Goolish, E. M., Evans, R., Okutake, K. and Max, R. (1998). Chamber volume requirements for reproduction of the zebrafish *Danio rerio*. *Prog. Fish-Cult.* **60**, 127-132.

Gronquist, D. and Berges, J. A. (2013). Effects of aquarium-related stressors on the zebrafish: a comparison of behavioral, physiological, and biochemical indicators. *J Aquat. Anim. Health* **25**, 53-65.

Hattori, R. S., Fernandino, J. I., Kishii, A., Kimura, H., Kinno, T., Oura, M., Somoza, G. M., Yokota, M., Strüssmann, C. A. and Watanabe, S. (2009). Cortisol-induced masculinization: Does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination? *PLoS ONE* **4**, e6548.

Hayashi, Y., Kobira, H., Yamaguchi, T., Shiraishi, E., Yazawa, T., Hirai, T., Kamei, Y. and Kitano, T. (2010). High temperature causes masculinization of genetically female medaka by elevation of cortisol. *Mol. Reprod. Dev.* **77**, 679-686.

Hazlerigg, C. R. E., Lorenzen, K., Thorbek, P., Wheeler, J. R. and Tyler, C. R. (2012). Density-dependent processes in the life history of fishes: evidence from laboratory populations of zebrafish *Danio rerio*. *PLoS ONE* **7**, e37550.

Herrera, M., Ruiz-Jarabo, I., Vargas-Chacoff, L., de la Roca, E. and Mancera, J. M. (2015). Metabolic enzyme activities in relation to crowding stress in the wedge sole (*Dicologoglossa cuneata*). *Aquacult. Res.* **46**, 2808-2818.

Holm, J. C., Refstie, T. and Bø, S. (1990). The effect of fish density and feeding regimes on individual growth-rate and mortality in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **89**, 225-232.

Howe, K. Clark, M. D. Torroja, C. F. Torrance, J. Berthelot, C. Muffato, M. Collins, J. E. Humphray, S. McLaren, K. Matthews, L. et al. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496**, 498-503.

Huang, W.-B. and Chiu, T.-S. (1997). Effects of stocking density on survival, growth, size variation, and production of Tilapia fry. *Aquac. Res.* **28**, 165-173.

Huertas, M. and Cerdà, J. (2006). Stocking density at early developmental stages affects growth and sex ratio in the European Eel (*Anguilla anguilla*). *Biol. Bull.* **211**, 286-296.

Iguchi, K., Ogawa, K., Nagae, M. and Ito, F. (2003). The influence of rearing density on stress response and disease susceptibility of ayu (*Plecoglossus altivelis*). *Aquaculture* **220**, 515-523.

Kleppe, L., Karlsen, Ø., Edvardsen, R. B., Norberg, B., Andersson, E., Taranger, G. L. and Wargelius, A. (2013). Cortisol treatment of prespawning female cod affects cytogenesis related factors in eggs and embryos. *Gen. Comp. Endocrinol.* **189**, 84-95.

Kotani, T., Wakiyama, Y., Imoto, T., Suzuki, H. and Fushimi, H. (2009). Effect of initial stocking density on larviculture performance of the ocellate puffer, *Takifugu rubripes*. *J. World Aquacult. Soc.* **40**, 383-393.

Krueger, W. H. and Oliveira, K. (1999). Evidence for environmental sex determination in the American eel, *Anguilla rostrata*. *Environ. Biol. Fishes* **55**, 381-389.

Kuwamura, T., Kadota, T. and Suzuki, S. (2014). Testing the low-density hypothesis for reversed sex change in polygynous fish: experiments in *Labroides dimidiatus*. *Sci. Rep.* **4**, 4369.

Larson, E. T., O'Malley, D. M. and Melloni, R. H. (2006). Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. *Behav. Brain Res.* **167**, 94-102.
Lawrence, C. (2007). The husbandry of zebrafish (*Danio rerio*): a review. *Aquaculture* **269**, 1-20.
Lawrence, C., Ebersole, J. P. and Kesseli, R. V. (2008). Rapid growth and out-crossing promote female development in zebrafish (*Danio rerio*). *Environ. Biol. Fishes* **81**, 239-246.
Leach, G. J. and Taylor, M. H. (1980). The role of cortisol in stress-induced metabolic changes in *Fundulus heteroclitus*. *Gen. Comp. Endocrinol.* **42**, 219-227.
Liew, W. C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K. R. and Orban, L. (2012). Polygenic sex determination system in zebrafish. *PLoS ONE* **7**, e34397.
Lisansky, J., Peake, G. T., Strassman, R. J., Qualls, C., Meikle, A. W., Risch, S. C., Fava, G. A., Zownirbrazis, M., Hochla, P. and Britton, D. (1989). Augmented pituitary corticotropin response to a threshold dosage of human corticotropin-releasing hormone in depressives pretreated with metyrapone. *Arch. Gen. Psychiat.* **46**, 641-649.
Lutnesky, M. M. F. (1994). Density-dependent protogynous sex-change in territorial haremic fishes—models and evidence. *Behav. Ecol.* **5**, 375-383.
MacKenzie, S., Montserrat, N., Mas, M., Acerete, L., Tort, L., Krasnov, A., Goetz, F. W. and Planas, J. V. (2006). Bacterial lipopolysaccharide induces apoptosis in the trout ovary. *Reprod. Biol. Endocrinol.* **4**, 46.
Mankiewicz, J. L., Godwin, J., Holler, B. L., Turner, P. M., Murashige, R., Shamey, R., Daniels, H. V. and Borski, R. J. (2013). Masculinizing effect of background color and cortisol in a flatfish with environmental sex-determination. *Integr. Comp. Biol.* **53**, 755-765.
Mayden, R. L., Tang, K. L., Conway, K. W., Freyhof, J., Chamberlain, S., Haskins, M., Schneider, L., Sudkamp, M., Wood, R. M., Agnew, M., et al. (2007). Phylogenetic relationships of Danio within the order cypriniformes: a framework for comparative and evolutionary studies of a model species. *J. Exp. Zool. B Mol. Dev. Evol.* **308**, 642-654.
McGonnell, I. M. and Fowkes, R. C. (2006). Fishing for gene function-endocrine modelling in the zebrafish. *J. Endocrinol.* **189**, 425-439.
Miranda, C. L., Henderson, M. C. and Buhler, D. R. (1998). Evaluation of chemicals as inhibitors of trout cytochrome P450s. *Toxicol. Appl. Pharm.* **148**, 237-244.
Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* **9**, 211-268.
Moretz, J. A., Martins, E. P. and Robison, B. D. (2007). The effects of early and adult social environment on zebrafish (*Danio rerio*) behavior. *Environ. Biol. Fishes* **80**, 91-101.
North, B. P., Turnbull, J. F., Ellis, T., Porter, M. J., Migaud, H., Bron, J. and Bromage, N. R. (2006). The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **255**, 466-479.
O'Connor, C. M., Gilmour, K. M., Arlinghaus, R., Matsumura, S., Suski, C. D., Philipp, D. P. and Cooke, S. J. (2011). The consequences of short-term cortisol elevation on individual physiology and growth rate in wild largemouth bass (*Micropterus salmoides*). *Can. J. Fish. Aquat. Sci.* **68**, 693-705.
OECD. (2011). Guideline for the testing of chemicals. Test number: 234: Fish sexual development test. . Available at: http://www.oecd-ilibrary.org/environment/test-no-234-fish-sexual-development-test_9789264122369-en
Pavlidis, M., Digka, N., Theodoridi, A., Campo, A., Barsakis, K., Skouradakis, G., Samaras, A. and Tsalafouta, A. (2013). Husbandry of zebrafish, *Danio rerio*, and the cortisol stress response. *Zebrafish* **10**, 524-531.
Penman, D. J. and Piferrer, F. (2008). Fish gonadogenesis. Part I: Genetic and environmental mechanisms of sex determination. *Rev. Fish. Sci.* **16**, 16-34.
Piferrer, F., Baker, I. J. and Donaldson, E. M. (1993). Effects of Natural, synthetic, aromatizable, and nonaromatizable androgens in inducing male sex differentiation in genotypic female Chinook Salmon (*Oncorhynchus tshawytscha*). *Gen. Comp. Endocrinol.* **91**, 59-65.
Poursaeid, S., Falahatkar, B., Mojazi Amiri, B. and Van der Kraak, G. (2012). Effects of long-term cortisol treatments on gonadal development, sex steroids levels and ovarian cortisol content in cultured great sturgeon *Huso huso*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **163**, 111-119.
Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L. and Schreck, C. B. (2006). Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture* **258**, 565-574.
Ribas, L. and Piferrer, F. (2014). The zebrafish (*Danio rerio*) as a model organism, with emphasis on applications for finfish aquaculture research. *Rev. Aquacult.* **6**, 209-240.
Ribas, L., Liew, W. C., Díaz, N., Sreenivasan, R., Orbán, L., and Piferrer, F. (2017). Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes. *Proc. Natl. Acad. Sci. USA* **114**, E941-E950.
Roncarati, A., Melotti, P., Mordenti, O. and Gennari, L. (1997). Influence of stocking density of European eel (*Anguilla anguilla*, L.) elvers on sex differentiation and zootechnical performances. *J. Applied Ichthyol.* **13**, 131-136.
Saillant, E., Fostier, A., Haffray, P., Menu, B., Laureau, S., Thimonier, J. and Chatain, B. (2003). Effects of rearing density, size grading and parental factors on sex ratios of the sea bass (*Dicentrarchus labrax L.*) in intensive aquaculture. *Aquaculture* **221**, 183-206.
Schreck, C. B. (1981). Stress and compensation in teleostean fishes: response to social and physical factors. In *Stress and Fish* (ed. A. D. Pickering), pp. 295-321. London: Academic Press.
Spence, R. and Smith, C. (2005). Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish, *Danio rerio*. *Anim. Behav.* **69**, 1317-1323.
Streisinger, G., Walker, C., Dower, N., Knauber, D. and Singer, F. (1981). Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature* **291**, 293-296.
Trevarrow, B. (2004). Zebrafish facilities for small and large laboratories. *Methods Cell Biol.* **77**, 565-591.
Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* **77**, 591-623.
Westerfield, M. (1995). *The Zebrafish Book. A Guide for the Laboratory use of Zebrafish (Danio Rerio).* Eugene, Oregon, USA: University of Oregon Press.
Whitfield, T. T., Granato, M., vanEeden, F. J. M., Schach, U., Brand, M., FurutaniSeiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J. et al. (1996). Mutations affecting development of the zebrafish inner ear and lateral line. *Development* **123**, 241-254.
Wilson, C. A., High, S. K., McCluskey, B. M., Amores, A., Yan, Y. L., Titus, T. A., Anderson, J. L., Batzel, P., Carvan, M. J., III, Schartl, M. et al. (2014). Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. *Genetics* **198**, 1291-1308.
Yamaguchi, T., Yoshinaga, N., Yazawa, T., Gen, K. and Kitano, T. (2010). Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. *Endocrinol.* **151**, 3900-3908.
Zanuzzo, F. S. and Urbinati, E. C. (2015). Dietary metyrapone blocks cortisol synthesis in pacu, *Piaractus mesopotamicus* (Holmberg, 1887), stressed by air exposure. *J. Appl. Ichthyol.* **31**, 1093-1095.