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ORIGINAL PAPER

Effects of social isolation on growth, stress response, and immunity of zebrafish

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Abstract Stressful housing conditions like social isolation have been shown to profoundly affect the physiology and health of various organisms which is rarely addressed in fish species. In the present study, we used a shoaling species, zebrafish, to investigate the stress reactivity of grouped and individually housed fish. We also hypothesized if isolation is a stressful condition may disrupt growth performance and innate immune response of individuals. To this end, fish were housed individually (social isolated treatment) or in groups of five fish (control treatment) for 60 days. Growth indices of fish were not affected by social isolation. Sixty-day social isolation did insignificant effect on baseline cortisol levels of specimens; however, individually housed zebrafish showed lower plasma cortisol to chasing stress than the control grouped fish. On the contrary, exposure to predator caused higher cortisol levels in social isolated fish. Serum lysozyme activity of isolated individuals was significantly lower than control fish, but activity of serum complement remained unchanged. Our results represent evidences that zebrafish experienced social isolation showed broad changes in physiological and immunological functions which may affect the quality of life.

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Introduction

Social isolation, or lack of social interactions among individuals (House 2001), represents a kind of psychosocial stress which exerts negative effects on the quality of life of the isolated individuals (Kanitz et al. 2004; Toth et al. 2011). For example, social isolation causes behavioral fragmentation and higher level of aggression in rats (Toth et al. 2011), increased aggression and established dominant-subordinate relationships in zebrafish, *Danio rerio* (Larson et al. 2006), and shorter survival time in mice with liver cancer (Liu and Wang 2005), while social deprivation during the early life causes disruption of brain development and behavioral changes that persist in adult rats (reviewed in Fone and Porkess 2008). Of course, individuals of group-living species have to percept the social status and the relationship with surrounding environment (Desjardins et al. 2012). Direct involvement in or indirect observation of social interactions among other co-specifics is the only ways by which individuals gain information about the social environment (McGregor and Peake 2000).

The effects of social isolation on stress have been addressed by studying the functional responses of the hypothalamic-pituitary-adrenal (HPA) or interrenal (HPI) axis in a range of species (Tuchscherer et al. 2004; Toth et al. 2011; reviewed in Serra et al. 2007) including zebrafish (Giacomini et al. 2015). Despite controversial results, these studies showed significant changes in blood cortisol concentrations as a main consequence of social isolation. Cortisol is involved in body homeostasis by influencing energy metabolism, growth, and the immune system (Bonga 1997). Yet, the stressful effects

of isolation on immune responses have been overlooked, even if the crosstalk between the neuroendocrine and immune systems mediated by cytokines is well known. In mammalian models of health, there are evidences that social isolation impairs the immune system (Kanitz et al. 2004; Tuchscherer et al. 2004; Bowers et al. 2008) and exposes isolated animals to disease (Bartolomucci 2007).

In teleost fish, on the other hand, the evaluation of social isolation effects on behavior has long been investigated (Halperin et al. 1992; Gómez-Laplaza and Morgan 2000; Larson et al. 2006). Only recently, some studies (Maruska et al. 2012; Galhardo and Oliveira 2014; Brandão et al. 2015) have dealt with the cognition, learning, and endocrine regulation of social isolation. As a highly social species, zebrafish have been increasingly used in behavioral studies (Larson et al. 2006; Pagnussat et al. 2013) and successfully employed to study mechanisms regulating social behaviors (Parker et al. 2012; Shams et al. 2015). Surprisingly, studies on zebrafish reported a reduction of cortisol concentrations in response to social isolation (Parker et al., 2012, Lindsey and Tropepe 2014; Giacomini et al. 2015), albeit Pagnussat et al. (2013) found higher cortisol levels in zebrafish individually exposed to a novel environment than in groups of three. However, to the best of our knowledge, there is no study on the immune response following isolation in fish. This represents an important issue because fish, in contrast to higher vertebrates, are equipped with a simpler innate immune defense system (Whyte 2007). Isolation act as a source of stress cause increase levels of anxiety and negatively affect the overall wellbeing (Huntingford et al. 2006), which may eventually depress immune responses. In fish, for example, has been showed that major indicators of innate immune system such as activities of lysozyme and alternative complement may change under stress and environmental conditions (Bowden 2008).

In this study, we used the zebrafish to address the following questions: (1) does social isolation during the development influences the stress system, growth, and immune responses? and (2) does social isolation impairs the HPI responses to acute external stressors? Both questions are relevant provided the relationships between stress, growth performance, and/or immune response in fish (Bonga 1997) and the divergent responses to variable stressors in laboratory settings (Pavlidis et al. 2013).

Materials and methods

Animals

Juvenile long-fin albino zebrafish ($n = 250$; 1 month old) were obtained from a local zebrafish hatchery. Fish were kept in two 95-L glass tanks (85 × 40 × 40 cm filled to the height of 30 cm) equipped with a sponge filter, a thermometer, and without any gravel on the bottom. They were fed twice daily (3% of body weight) to apparent satiation with commercial granular (BioMar group, Tehran, Iran, imported from France) and flake (Energy, Mahiran, Tehran, Iran) foods. Water was changed in part with fresh de-chlorinated tap water every 3 days. Fish were acclimated to the laboratory condition (~8 mg O_2/L; 27 ± 1 °C; pH 7.3–7.8; total hardness at 15–18 mg/L; 14 L:10D photoperiod) for 2 weeks prior to experiments. Animal handling and testing techniques were designed according to the guidelines provided by the Association for the Study of Animal Behavior and the Animal Behavior Society (ASAB/ABS 2012).

Experimental procedure

Control and a socially isolated (SI) treatment groups were designed to evaluate the influence of isolation on zebrafish. From the fish stock, 140 uniform-sized fish harvested using a plastic-mesh sorter were randomly assigned to either control or SI treatment (78.16 ± 5.09 and 79.43 ± 7.21 mg, respectively). The control fish were housed in groups of five into 14 opaque circular plastic containers (23 cm in diameter with 4 L water). The fish of the SI treatment were housed in 70 opaque circular plastic containers (10 cm diameter with 0.6 L water) with one fish in each container. Treatments lasted 60 days in accordance to previous studies on social deprivation in zebrafish (from 5 to 90 days; Larson et al. 2006; Shams et al. 2015). The physicochemical properties of water and laboratory conditions were those of the acclimation period.

Growth assessment

Fishes (20 per group) were randomly sampled for the evaluation of body weight at days 15, 30, 45, and 60 of the experiment. Once removed, the fish were slightly anesthetized in clove powder (30 s in 50 mg/L; Grush et al. 2004) and weighted on a digital balance with 0.001 g accuracy. After recovery in fresh water, the fish were located back into their respective tanks. We calculated the amount of food requirements (3% body weight) for each group based on previous 15-day interval body weight measurements. Final body weight, specific growth rate (SGR), and feed conversion ratio (FCR) were assessed as the fish growth performance.

Stress response assay

At the end of the experiment, 10 fish per treatment were randomly sampled for evaluating cortisol concentrations as a marker of chronic response to social isolation stress. Another 30 fish per treatment were randomly assigned to the stress induction test and used for evaluating the cortisol concentrations as a marker of acute stress. We applied two standard methods for the acute stress induction test: (i) chasing (de

Abreu et al. 2014) or (ii) exposure to a predator (Barcellos et al. 2007). All fish were fasted 12 h before the stress induction tests.

For the chasing stress test, 20 fish per group were chased in their home tanks (4 and 20 for control and SI treatments, respectively) with a net for 2 min. Cortisol levels were sampled 15 min after the stress challenge (Giacomini et al. 2015).

For the predator exposure test, two groups of five fish (control treatment) or 10 fish (SI treatment) were separately exposed to a model predator, convict cichlid (*Amatitlania nigrofasciata*), in test tanks. To acclimate with the new environment, 24 h before the exposure test, the zebrafish and the predators were located in test tanks (60 × 20 × 20 cm, 18 L) which were divided in two parts by a transparent glass. An opaque plastic sheet mounted on the divider prevented visual contact between subjects and predators. The predator exposure stress test started after removing the opaque sheet. After 45-min exposure, the opaque sheet was located back into the tank and the fish were sampled for cortisol.

Cortisol was extracted from the whole body according to the method described by Barcellos et al. (2007). Fish were euthanized with 500 mg/L of clove powder and immediately frozen at −20 °C. Each fish was then weighed, sliced into small sections, and a pool of two fish were minced and placed into a disposable stomacher bag with 2 ml of phosphate-buffered saline (PBS, pH 7.4) for 6 min. The contents were transferred to a 15-ml screw top test tube and 5 ml of laboratory grade diethyl ether was added. The tube was vortexed for 1 min followed by centrifuging at 3000×*g* for 10 min. The tube was then immediately frozen at −20 °C for 2 h, and the unfrozen portion was decanted into a fresh 15-ml test tube. The diethyl ether was evaporated under a flow of air, and cortisol was reconstituted in 1 ml PBS. The extracts were stored at −80 °C until cortisol concentrations were assayed by using a commercial ELISA kit according to the manufacturer's instructions (AccuBind™ microplate EIA, Monobind Inc., USA). The inter- and intra-assay coefficient of variance was 8.2 and 7.7%, respectively, which were determined by measuring three known cortisol concentrations with five replications. The concentrations of cortisol were measured in duplicate samples of tissue extract in a single plate.

Immune response assay

At the end of the experiment, 30 fish per group were euthanized with 500 mg/L of clove powder. By severing the caudal vein, blood samples were immediately collected via a 10 μl micro-sampler into a 0.5-ml Eppendorf tube. Samples of 7–8 fish were pooled as a replicate to obtain four replicates per treatment. Blood samples were centrifuged, at 1500×*g* for 10 min at 4 °C, and serum was collected and stored at −20 °C.

Lysozyme activity was assayed according to the method of Swain et al. (2007). Lyophilized *Micrococcus lysodeikticus* cells (Razi Vaccine and Serum Research Institute, Karaj, Iran) were dissolved at a concentration of 0.2 mg/ml in 0.02 M sodium citrate buffer. The resulting solution was added to the serum samples at 10:1. Initial OD was taken at 450 nm immediately after adding *M. lysodeikticus*. The second OD was taken after 1 h incubation at 25 °C. Standard curve was developed using lyophilized hen egg white lysozyme (HEWL: Sigma). Serum lysozyme values were expressed as microgram per milliliter equivalent of HEWL activity.

The serum alternative complement activity was determined after Ortuno et al. (1998). For each replicate, 25 μl of serum was used. The hemolytic activity was measured using washed sheep red blood cells as targets cells in the presence of EGTA and Mg^{2+}. The volume of serum yielding 50% hemolysis was determined and used to calculate the complement activity of each sample (Yarahmadi et al. 2016).

Statistics

We checked for homogeneity of variance with Levene's test in all statistical tests. Shapiro-Wilk test was also used to assess normality assumption of the data. Independent-samples *t* tests were used to compare the growth indices between treatments. Also, weight of the subjects were analyzed over the 60 days experiment using a mixed model ANOVA with treatments as the between-subject effect and fish weight at 15-day intervals as within-subject variables. The concentrations of cortisol responses to social status were arcsine square-root transformed prior to submitting to independent-samples *t* test. In addition, cortisol levels after stress tests, and immune parameters were compared between treatment using independent-samples *t* tests. Data are reported as mean ± SE and all the statistical analyses were conducted using SPSS (version 22.0; IBM Statistics) at the significant level of $p < 0.05$.

Results

The fish in the present study showed no mortality during the 60-day experiment or in response to the stress induction test. There was no significant difference in the initial body weight of the fish among treatments (Table 1). Similar results were found for the final body weight, specific growth rate, and food conversion ratio at the end of experiment (Table 1). These findings confirm that social isolation has no effect on zebrafish growth performance. Furthermore, the mixed model ANOVA showed that treatments did not affect the weight of fish at 15-day intervals ($F_{1,38} = 0.34$; $p = 0.56$), but it increased during the course of experiment ($F_{1,38} = 932.53$; $p < 0.001$), although there was no effects of treatment × growth at 15-day intervals ($F_{1,38} = 0.36$; $p = 0.54$).

Table 1 Growth performance of control (group-housed) and socially isolated (individually housed) zebrafish after 60 days experiment. Values are mean ± SE

Growth endpoint	Treatment		*t*	df	Sig.
	Control	Social isolation			
Initial weight (mg)	68.16 ± 1.14	69.43 ± 1.11	0.28	38	0.817
Final weight (mg)	227.76 ± 5.78	223.40 ± 6.05	0.52	38	0.605
SGR[a]	1.67 ± 0.04	1.61 ± 0.05	0.97	38	0.333
FCR[b]	1.38 ± 0.24	1.43 ± 0.22	0.14	6	0.889

[a] Specific growth rate = (Ln final weight − Ln initial weight) × 100 / 60 days

[b] Feed conversion ratio = dry feed fed (mg) / wet weight gain (mg)

Results of the stress responses to social status showed that isolation lasting 60 days did not influence the whole-body cortisol concentrations of the zebrafish (9.03 ± 0.49 vs. 8.69 ± 0.50 ng/g body weight, $p = 0.628$).

Following the chasing stress test, social isolated fishes showed lower ($p < 0.05$) whole-body cortisol concentrations when compared to the control treatment (Fig. 1a). By converse, the predator stress test increased ($p < 0.05$) cortisol concentrations of socially isolated fish compared to those of the control treatment (Fig. 1b).

Social isolation lasting 60 days reduced ($p < 0.05$) lysozyme activity in comparison to the control treatment (Fig. 2a), but did not influence ($p = 0.072$) serum concentrations of the complement activity (Fig. 2b).

Discussion

We show here that social isolation influences the immune function in a social fish species but does not affect growth indices. In addition, fish subjected to social isolation showed an opposite cortisol secretion pattern in response to the different stressful conditions.

Growth is a complex process regulated by several internal and external factors. The amount of food consumption is one of the main factors relating to growth performance. However, it is now well established how much environmental and/or social stressors may negatively affect food intake and growth of fish (reviewed in Bernier and Peter 2001). For example, reduction in food intake is a behavioral response to stress in fish species (Bonga 1997). Social isolation is also a stressful condition itself (House 2001; Hermes et al. 2011), and hence, we had expected lower food intake and growth rate in the isolated subjects. However, in our study, fish showed a similar food conversion ratio during the experiment and, therefore, it

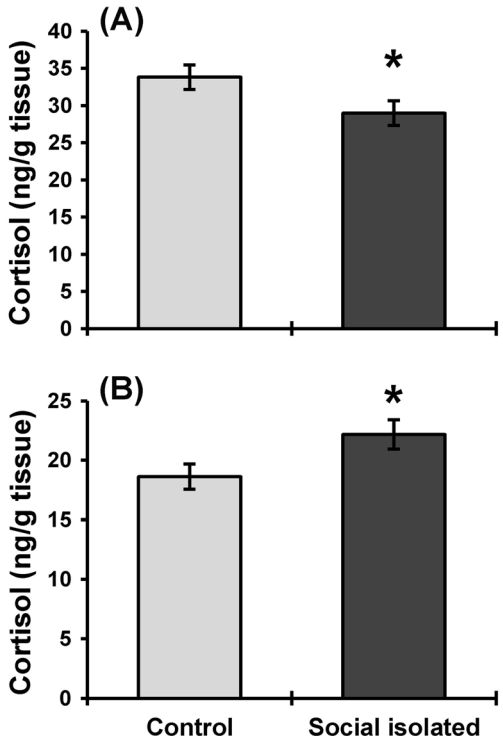

Fig. 1 Whole-body cortisol concentrations (ng/g) in control (group-housed) and socially isolated (individually housed) zebrafish (mean ± SE) after chasing stress (**a**) and predator exposure stress (**b**) tests; *$p < 0.05$

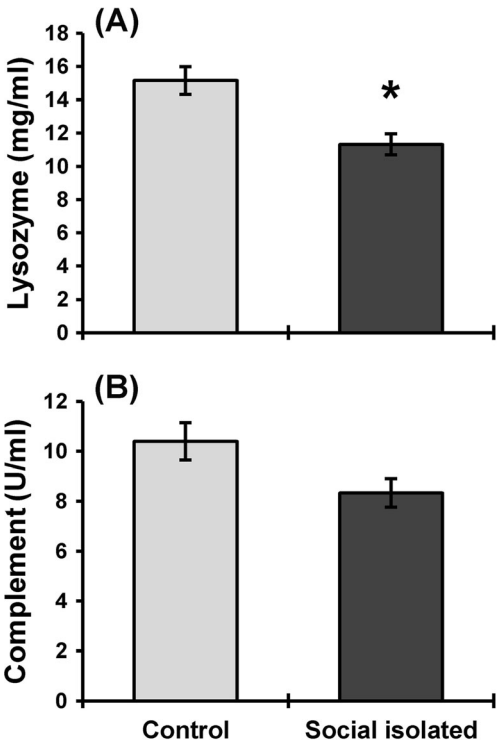

Fig. 2 Serum activity levels (mean ± SE) of lysozyme (**a**) and alternative complement (**b**) in control (group-housed) and socially isolated (individually housed) zebrafish; *$p < 0.05$

is not surprising that they grew to similar weight. This unexpected finding suggests that the protocol here adopted for social isolation does not represent a stressful condition in fish as indirectly confirmed by the similar cortisol concentrations found in socially isolated and control treatments. We cannot rule out, however, that in fish there are complex brain regulatory mechanisms of food intake modulated by the environment (Lin et al. 2000; Bernier and Peter 2001) which remains to be investigated in future studies.

Results of the present study showed that stressors, like chasing and predator exposure, induced a differential cortisol response in fish. In socially isolated fish, chasing lowered, while predator exposure increased whole-body cortisol levels. However, social isolation per se did not influence baseline cortisol concentrations that were superimposable to those of controls. These results are somewhat surprising if we consider that social isolation, being a well-known stressful condition, should have affected baseline cortisol values similarly to other stressors. No significant change in cortisol responses was reported in a recent study on zebrafish isolated for 15 and 30 days (Giacomini et al. 2015). Quite surprisingly, in another study by Parker et al. (2012), socially isolated zebrafish had lower baseline cortisol levels than group-housed fish. Our results and the previous ones, therefore, suggest that social isolation is not a stressful factor as it was thought, at least in zebrafish living under laboratory condition. By converse, the higher cortisol levels found in control fish exposed to chasing stress may be related to the "group effect" that has been demonstrated in fish (Ramsay et al. 2006; Parker et al. 2012; Giacomini et al. 2015) and other taxa (de Haas et al. 2012; Schoepf and Schradin 2013). The group effect is defined as the interactions between individuals, either chemical or behavioral, which can alter behavior and activate the HPI axis (Barcellos et al. 2007). In a recent study, Faustino et al. (2017) found that zebrafish in the presence of olfactory and visual cues of conspecifics decreased fear response to alarm substances, thus confirming that the mere availability of conspecifics may reduce behavioral and physiological responses to adverse events. On the other hand, socially isolated fish exposed to predator did not comply with the group effect and showed a higher cortisol response compared to that of control fish. These findings indicate that different mechanisms are activated by each stressor. Fish prefer a group of conspecifics and form a school when a predator is presented (Krause et al. 1998); accordingly, the grouping behavior seems to be advantageous for each individual. For example, they may gain benefits from the social support (Krause and Godin 1994), or share their responses to successfully escape from the predator (Magurran et al. 1985). Therefore, it may reasonable to note that isolated zebrafish were more scared of the predator, and thus elevated stress response in the form of cortisol levels. It is worth noting that the statistic here falls around the pseudoreplication, which is an incorrect modeling of randomness in experiments (Millar and Anderson 2004). However, when the precise details of an experiment are well known, common sense, biological knowledge, and intuition should be applied to that task (Dal Bosco et al. 2014). The conditions in the present study were almost identical, and then, it is reasonable to consider the presented results.

Among innate defense parameters, lysozyme and complement have crucial functions in the immune system of fish (Whyte 2007; Wang and Zhang 2010). In studies on stress response, measurements of serum lysozyme and alternative complement activities are suitable indicators of immune capability (Tort et al. 1996; Yarahmadi et al. 2016). It has been showed that stressful housing condition may have adverse effects on the status of innate immune system of various fish species (Wang and Zhang 2010; Yarahmadi et al. 2016). In the present study, lysozyme activity was lower in socially isolated fish, but complement activity remained unaffected. Although no information is available on the modulating effects of social isolation in fish, our results suggest that isolated zebrafish are more prone to the risk of immune suppression. In agreement with our results, previous studies on mammals showed alterations in immunologic functions and even higher mortality in response to social isolation (Kanitz et al. 2004; Tuchscherer et al. 2004; Liu and Wang 2005). However, it remains to find out the mechanisms underlying these changes in the immune system of the zebrafish as well as of other aquatic organisms.

In summary, the present study showed the variable effects of social isolation on growing zebrafish. Individually isolated fish grew similarly to group-housed fish and showed no difference in FCR. After 60 days of treatment, baseline cortisol levels were similar in the both isolated and group-housed fish; however, cortisol concentration of socially isolated fish was lower after chasing stress and higher after predator exposure stress compared to control fish. Furthermore, lysozyme activity in socially isolated fish was significantly lower than controls, while alternative complement activity did not differ among treatment groups, despite it was lower in the isolated fish. Further studies are needed to address the mechanisms underlying these differential responses to different stressors. Thus, this study may be a start of the investigations dealing with physiological and molecular aspects of social isolation using the fish as model species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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