South African Journal of Animal Science 2021, 51 (No. 5)

Response of zebrafish to royal jelly supplementation and differences in stocking density

B.S. Pires, D.A.B. Moreski, F.R. Domanski, M.R. Sippert, A.S. Khatlab, R.P. Ribeiro, L.D. Castilha & E. Gasparino[#]

Department of Animal Science, State University of Maringá, Avenida Colombo, 5790, Maringá, Paraná State, 87.020-900, Brazil

(Received 15 December 2020 Accepted 13 June 2021 Published 30 September 2021)

Abstract

Dietary supplementation with royal jelly (RJ) may improve growth, antioxidant gene expression, and intestinal antioxidant capacity, and possibly mitigate effects of stress induced by high stocking density (HSD) in zebrafish *(Danio rerio).* Effects of HSD and RJ supplementation on growth performance, antioxidant gene expression, and intestinal antioxidant capacity in zebrafish were investigated. A total of 240 four-month-old zebrafish were used in a 2 x 2 factorial design, with four treatments and three replications. The treatments consisted of normal stocking density (NSD) (1 fish/L) and high stocking density (HSD) (3 fish/L) and an unsupplemented commercial diet (0RJ) and commercial diet supplemented with 10% RJ (10RJ). The experiment lasted 25 days. Daily weight gain, final body length, specific growth rate, and survival were recorded. On day 25, six fish per treatment were euthanised and their intestines analysed for antioxidant capacity, and superoxide dismutase 2 *(SOD2)* and catalase *(CAT)* gene expression. There were no effects of diet or SD and diet interaction on growth. High stocking density decreased daily weight gain by 25.31%, body length by 2.70%, and survival by 16.85%. Stocking density (SD) and diet interaction influenced intestinal SOD2 and CAT expression and antioxidant capacity. The HSD/0RJ group had lower antioxidant gene expression and antioxidant capacity than the HSD/10RJ and NSD/0RJ groups. Supplementation with RJ did not mitigate the harmful effects of stocking stress. These results indicate that HSD promotes transcriptional suppression of gene encoding enzymes that form the first line of antioxidant defence, resulting in reduced elimination of toxic substances and occurrence of intestinal oxidative stress.

Keywords: antioxidant capacity, antioxidant gene expression, growth performance, intestinal environment, overcrowding, stress

[#]Corresponding author: gasparinoeliane@gmail.com

Introduction

Animal production efficiency depends on a variety of factors, including the environment in which animals are bred, because genes associated with economically important traits are influenced by the environment (Côté *et al.*, 2007). In fish farming, stocking density (SD) is a highly relevant environmental factor, affecting water quality, animal wellbeing, uniformity, and health (Marques *et al.*, 2004; Ashley, 2007; Dawood *et al.*, 2019). An inadequately high SD may alter feeding behaviour and feed use efficiency, thereby decreasing productive performance (Wocher *et al.*, 2011; Rabbane *et al.*, 2016; Refaey *et al.*, 2018; Long *et al.*, 2019). Highly stocked fish may develop a state of physiological stress in response to increased risk of predation and competition for food (Bolasina *et al.*, 2006; Jia *et al.*, 2016; Long *et al.*, 2019). Under these conditions, metabolic energy is directed towards stress management rather than growth.

Stress can also lead to short- and long-term alterations in gastrointestinal tract functions, exerting negative effects on microflora, intestinal mucosa blood flow, regenerative capacity, and gastrointestinal motility and secretion (Konturek *et al.*, 2011). Previous studies showed that fish raised in highly stocked environments had altered length and bodyweight (Bolasina *et al.*, 2006; Liu *et al.*, 2016) and metabolic changes such as increased cortisol levels (Bolasin *et al.*, 2006; Ramsay *et al.*, 2006; Long *et al.*, 2019) and altered immune response (Montero *et al.*, 1999; Liu *et al.*, 2016; Dawood *et al.*, 2019). Another known effect of high stocking density (HSD) on fish is the occurrence of oxidative stress (Jia *et al.*, 2016; Liu *et al.*, 2016;

URL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v51i5.6

Dawood *et al.*, 2019), caused by an increase in lipid peroxidation and a reduction in the activity of antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase) and levels of the non-enzymatic antioxidant glutathione (Liu *et al.*, 2016; Dawood *et al.*, 2019).

In intensive fish farming, productivity is optimized by using high breeding densities (Bolasina *et al.* (2006). An option to minimize the negative effects of stress on fish grown under these conditions is to supplement feed with bioactive ingredients. For instance, royal jelly (RJ), a honeybee secretion, is considered a nutraceutical food with anti-inflammatory (Aslan & Aksoy, 2015), antimicrobial (Coutinho *et al.*, 2018), antiallergic (Guendouz *et al.*, 2017), antioxidant (Nagai *et al.*, 2006), and growth-stimulating properties (Ahangari *et al.*, 2013). This study aimed to investigate the hypotheses that HSD (SD) causes oxidative stress in the intestine of zebrafish *(Danio rerio)* and that RJ supplementation is able to mitigate the harmful effects of stocking stress on the intestine. To date, this is the first study to simultaneously evaluate the effect of high SD and RJ supplementation on zebrafish performance (weight gain, final body length, specific growth rate, and survival), expression of genes involved in antioxidant defences (superoxide dismutase and catalase genes), and intestinal antioxidant capacity.

Material and Methods

This study was conducted according to the guidelines of the Animal Ethics Committee of the State University of Maringá, Brazil. A total of 240 four-month-old zebrafish *(D. rerio)* were used with 0.33 ± 0.11 g bodyweight and 3.35 ± 0.20 cm total length. The experiment was conducted at PeixeGen Zebrafish Laboratory of the State University of Maringá, Brazil, following a completely randomized design arranged in a 2 × 2 factorial, with three replications per treatment. Treatments consisted of two SDs, namely NSD (1 fish/L) and HSD (3 fish/L) and two diets, an unsupplemented commercial diet (0RJ) and a commercial diet supplemented with 10% RJ (10RJ). Stocking densities were chosen according to the recommendations of Vargesson (2007) and the observations of Ramsay *et al.* (2006).

Fish were randomly distributed in 10 L glass aquaria with chlorine-free water and constant aeration under controlled temperature conditions (28.5 ± 1 °C) and a photoperiod of 14 hours light/10 hours dark. Water quality parameters (temperature, ammonia, nitrite, and pH) were monitored weekly. To prevent other sources of stress and ensure water quality, the authors partially replaced water (60%) every two days. Fish were first maintained under conventional conditions for seven days. During this adaptation period, fish were fed commercial feed (without RJ supplementation) three times a day to apparent satiation (at 9h00, 14h00, and 17h00). After the adaptation period, fish were subjected to SD and diet treatments and fed three times a day (at 9h00, 14h00, and 17h00) for 25 days.

A commercial diet was used based on fish meal, pea, fish oil, soy lecithin and fish gelatine (Bernaqua Finest Hatchery Feeds, Belgium). Diet composition is presented in Table 1. The 10RJ diet was prepared by spraying a solution containing 100 mg pure freeze-dried RJ (Apireal, Apis, Flora, Brazil) diluted in 25 mL of distilled water onto the commercial diet. Then, kibbles were dried at room temperature (25 ± 4 °C) in the dark and stored in dark plastic containers at -22 °C until use.

At the beginning and end of the experiment, fish were anaesthetised in 0.0004% benzocaine (Medicinal Farmácia e Manipulação, Brazil) to the point of total loss of equilibrium and weighed. Daily weight gain was determined as the initial bodyweight minus the final bod weight divided by 25. Final body length was measured at the end of the experimental period. For this, fish were anaesthetised in benzocaine, and body length (from the beginning of the head to the end of the caudal fin) was measured using a digital calliper (model 366119, MXT, Brazil). Survival and specific growth rate were calculated as described by Gonzalez Jr. (2012).

For analysis of gene expression, three fish from each treatment (n = 3) were euthanized at the end of the experiment by immersion in ice for 5 min. The entire intestine was collected, washed with sterile saline (4 °C), frozen in liquid nitrogen, and stored at -80 °C until analysis. Total RNA was extracted from intestine specimens with TRIzol (Invitrogen, Waltham, Massachusetts, USA) according to the manufacturer's recommendations, and quantified using a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, USA) at 260 nm. Total RNA purity was determined by the ratio of absorbance readings at 260/280 nm and 260/230 nm, which fell between 1.9 and 2.1. Genomic DNA contamination was eliminated by treating 1 µg of total RNA with the amplification grade DNase I kit (Invitrogen, USA), according to the manufacturer's instructions. After this procedure, complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen Corporation, Brazil), according to the manufacturer's instructions. cDNA concentration was measured using a spectrophotometer at 260 nm (Nanodrop 2000c, Thermo Fisher Scientific, USA), and samples were stored at -20 °C until amplification.

Table 1 Composition of experimental diets fed to zebrafish *(Danio rerio)*

Composition	Commercial diet	Diet supplemented with 10% royal jelly
Protein (%)	60.0	60.0
Lipid (%)	15.0	15.0
Royal jelly (Apireal®) (%)	0.0	10.0
Omega-3 highly unsaturated fatty acids (mg/g)	25.0	25.0
Docosahexaenoic acid (mg/g)	10.0	10.0
Eicosapentaenoic acid (mg/g)	12.0	12.0
Fibre (%)	1.7	1.7
Vitamin A (IU/g)	2,500.0	2,500.0
Vitamin D3 (IU/g)	200.0	200.0
Vitamin C (mg/kg)	75.0	75.0
Vitamin E (mg/kg)	25.0	25.0
Calcium (%)	2.2	2.2
Phosphorus (%)	2.3	2.3
Sodium (%)	0.5	0.5
Ash (%)	14.5	14.5
Moisture (%)	8.0	8.0
Metabolizable energy (kJ/g)	18.6	18.6

Real-time quantitative polymerase chain reaction (qPCR) was performed using 5 µL of cDNA at 40 ng/mL, 0.8 µL of each primer (forward and reverse) at 10 µM (final concentration of 400 µM), 10 µL of PowerUp SYBR GREEN Master Mix (Applied Biosystems, Lithuania), and UltraPure DEPC-treated Water (Invitrogen, USA) to complete a final volume of 20 µL. All analyses were carried out on a StepOne Real-Time PCR System version 2.3 (Applied Biosystems, Lithuania), in duplicate, under thermal cycling conditions, namely activation of uracil-DNA glycosylase at 50 °C for 2 min, activation of Dual-Lock DNA-polymerase at 95 °C for 2 min, 40 cycles of 95 °C for 3 s for denaturation, and annealing and extension at 60 °C for 30 s. Melting curves were obtained from 60 to 95 °C.

Primers for superoxide dismutase 2 (*SOD2*) and catalase (*CAT*) genes of *D. rerio* were designed according to Sarkar *et al.* (2014) on the basis of gene sequences deposited at the National Centre for Biotechnology Information (NCBI) database (NCBI Resource Coordinators, 2018) (Table 2). The β-actin gene was used as endogenous control, and its primers were designed based on sequences deposited at NCBI using the Integrated DNA Technologies platform ~~(www.idtdna.com)~~ (Table 2). All analyses were performed in duplicate. The $2^{-\Delta CT}$ method (Livak & Schmittgen, 2001) was used for relative quantification of gene expression, and results are presented in arbitrary units (AU).

Table 2 Primers used for real-time polymerase chain reactions

Target gene[1]	Amplicon size (bp)	Annealing temperature	Primer sequence (5′→3′)	Accession number
SOD2	166	60 °C	F: AGCGTGACTTTGGCTCATTT R: ATGAGACCTGTGGTCCCTTG	NM_199976.1
CAT	126	60 °C	F: CTCCTGATGTGGCCCGATAC R: TCAGATGCCCGGCCATATTC	AF170069.1
β-actin	130	60 °C	F: ACCCCAAAGCCAACAGA R: CCAGAGTCCATCACAATACC	L08165.1

[1] *SOD2*, superoxide dismutase 2 gene; *CAT*, catalase gene; bp, base pairs; F, forward; R, reverse

Intestinal antioxidant capacity was determined in samples obtained from three fish per treatment (n = 3) at the end of the experimental period. Animals were stunned by immersion in ice for 5 min, and intestine samples were collected, washed with sterile saline (4 °C), frozen in liquid nitrogen, and stored at -80 °C until analysis. Antioxidant capacity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Sigma–Aldrich, Brazil), according to the protocol described by Brand-Williams *et al.* (1995), with some modifications. Briefly, intestine samples were added to a test tube containing 1000 µL of methanol and homogenized using a Van Potter homogeniser. The mixture was centrifuged for 10 min at 10,000 × g and 4 °C, and the supernatant (22.50 µL) was added to the wells of a 96-well microplate containing 277.50 µL of a 0.06 mM DPPH solution. Reactions were kept in the dark for 30 min. After this period, samples were read at 515 nm on a microplate reader (VersaMax, Molecular Devices, USA), and the antioxidant capacity (%) of each sample was determined as antioxidant capacity = (1 − (sample absorbance/DPPH absorbance)) × 100.

The Shapiro–Wilk test was used to assess the normality of data distribution. Main and interaction effects of SD and RJ supplementation were investigated by two-way analysis of variance (ANOVA). When significant, the main effect means were compared with Student's *t*-test and interaction means by Tukey's test. The level of significance was set at P <0.05. All statistical analyses were performed using SAS 2002 (SAS Inst. Inc., Cary, NC, USA).

Results and Discussion

No SD x diet effects were observed on weight gain, final body length, specific growth rate, or survival rate (P >0.05, Table 3). The main effects of SD on weight gain (P =0.0285), final body length (P =0.0475), and survival rate (P =0.0011) were significant. HSD led to a reduction of 25.31% in weight gain, 16.85% in survival rate, and 2.70% in final body length.

Table 3 Daily weight gains final body length, specific growth rate, and survival of zebrafish *(Danio rerio)* (1–25 days old) fed diets supplemented or not with royal jelly and reared at different stocking densities

Parameter	NSD		HSD		SD		Diet		SE	*P-value*		
	0RJ	10RJ	0RJ	10RJ	NSD	HSD	0RJ	10RJ		SD	RJ	SD × Diet
DWG (g)	0.26	0.24	0.19	0.18	0.25[a]	0.18[b]	0.22	0.21	0.04	0.029	0.518	0.918
FBL (cm)	3.77	3.66	3.62	3.61	3.71[a]	3.61[b]	3.66	3.62	0.33	0.048	0.418	0.289
SGR (mg/d)	1.76	2.31	1.90	1.78	2.04	1.84	1.83	2.04	0.31	0.267	0.304	0.102
Survival (%)	100.0	97.8	78.9	85.6	98.9[a]	82.2[b]	89.4	91.6	5.77	0.001	0.524	0.219

NSD: normal stocking density (1 fish/L), HSD: high stocking density (3 fish/L), SD: stocking density, RJ: royal jelly, 0RJ: unsupplemented commercial diet, 10RJ: commercial diet supplemented with 10% RJ, DWG: daily weight gain, FBL: final body length, SGR: specific growth rate

[a,b] Within a column, means with a common superscript did not differ with probability P <0.05

Figure 1 shows the *CAT* and *SOD2* expression and total antioxidant capacity in the intestine of zebrafish. There were significant effects of SD × diet on *CAT* expression (P <0.0001), *SOD2* expression (P =0.0006),

and antioxidant capacity (*P* <0.0001). Fish fed 0RJ and kept at HSD had reduced antioxidant gene expression and antioxidant capacity.

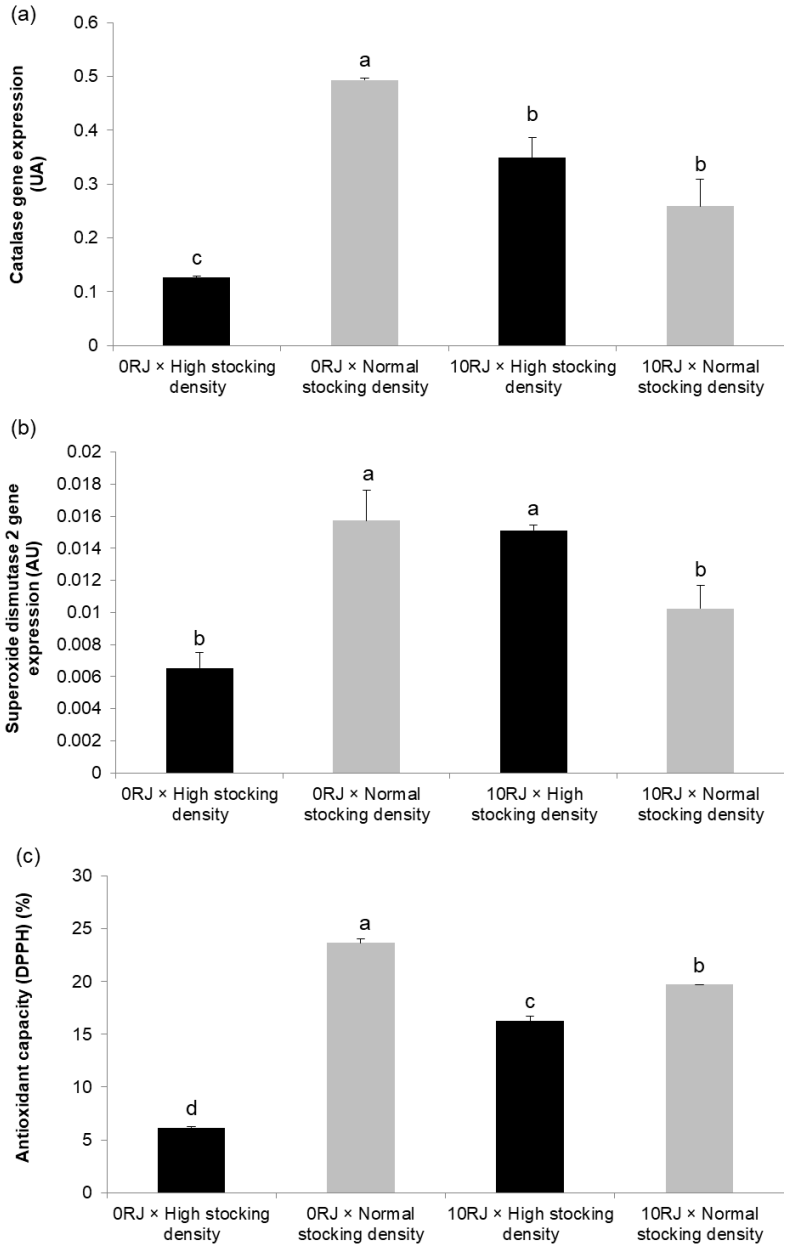

Figure 1 Effects of stocking density and royal jelly supplementation on a) catalase and b) superoxide dismutase 2 gene expression and c) antioxidant capacity in the intestine of zebrafish *(Danio rerio)*

Gene expression is presented in arbitrary units (AU). Values are the mean ± standard error of three replicate fish
Columns headed by a common letter did not differ with probability *P* <0.05
CAT: catalase; *SOD2*: superoxide dismutase 2
0RJ: unsupplemented commercial diet, 10RJ: commercial diet supplemented with 10% royal jelly

The current results indicated that the low performance of zebrafish reared at HSD might have occurred because of intestinal oxidative stress from transcriptional suppression of genes involved in antioxidant responses. Highly stocked zebrafish had lower weight gain, body length, and survival rate than zebrafish maintained under normal conditions, as in other studies (Costa *et al.*, 2017; Rabbane *et al.*, 2017; Refaey *et al.*, 2018; Dawood *et al.*, 2019; Long *et al.*, 2019). Stocking density is one of the most relevant environmental factors in the production of zebrafish and other fish, whether under laboratory or natural conditions (Hazlerigg *et al.*, 2012). Intensive fish farming systems are characterized by the use of high

breeding densities, a strategy for yield optimization (Bolasina *et al.*, 2006). However, when SD is inadequately high, important productive parameters may be affected (Costa *et al.*, 2017; Dawood *et al.*, 2019; Long *et al.*, 2019). The negative effects of HSD observed in the current study might have been owing to an increase in competition for food among animals, thereby leading to altered feeding behaviour (Wocher *et al.*, 2011) and nutrient use, ultimately decreasing productive performance. The findings may also have been owing to physiological and metabolic changes in response to stressful situations, such as increased cortisol secretion (Bolasina *et al.*, 2006; Ramsay *et al.*, 2006; Long *et al.*, 2019). High cortisol levels accelerate protein metabolism (van der Boon *et al.*, 1991) and increase the concentration of low molecular weight insulin-like growth factor (IGF)-binding proteins, which may inhibit IGF-1 activity (Peterson & Small, 2005).

When raised at HSDs, fish may develop a state of physiological stress characterized by numerous metabolic changes, including altered antioxidant response (Liu *et al.*, 2016; Dawood *et al.*, 2019). Such an effect can contribute to the establishment of oxidative stress (Dawood *et al.*, 2019). Given the widespread use of increasingly high SDs, it is necessary to find solutions to mitigate the harmful effects of stocking stress on physiological defence systems.

An interesting strategy for nutritional modulation of immune responses is the supplementation of animal feed with nutraceutical foods that have immunostimulatory, anti-inflammatory, and antioxidant properties (Taheri Mirghaed *et al.*, 2018; Herrera *et al.*, 2019; Bacchetta *et al.*, 2020). Various supplements, including amino acids, fatty acids, vitamins, minerals, and plant extracts, have been used to increase animal resistance to stress by modulating antioxidant and immune defence systems (Hoseinifar *et al.*, 2017; Taheri Mirghaed *et al.*, 2018; Herrera *et al.*, 2019; Bacchetta *et al.*, 2020). Royal jelly is a honeybee secretion with important anti-inflammatory (Aslan & Aksoy, 2015), antimicrobial (Coutinho *et al.*, 2018), antiallergic (Guendouz *et al.*, 2017), antioxidant (Nagai *et al.*, 2006), and growth-stimulating properties (Ahangari *et al.*, 2013). Despite the potential beneficial effects of this natural food on animal health, few studies have investigated the effect of supplementing animal diets with RJ (Seven *et al.*, 2012; Ferreira *et al.*, 2013; Seven *et al.*, 2016; Babaei *et al.*, 2016). Ferreira *et al.* (2013) assessed the effects of RJ supplementation on catfish exposed to a highly toxic fungicide and found that RJ and other bee products prevented or reversed oxidative tissue damage. The results were attributed to an increase in superoxide dismutase, catalase, and glutathione transferase activities. Aksakal *et al.* (2021) evaluated the effects of supplementing with RJ on the expression of several genes. The authors noted that RJ supplementation increased superoxide dismutase and catalase gene expression in the liver, muscle, and kidneys.

In this study, it was hypothesized that a high population density would cause oxidative stress in zebrafish intestines and that RJ supplementation would benefit the intestinal antioxidant defence system. To test this hypothesis, the authors evaluated gut antioxidant capacity and expression of two genes encoding enzymes regarded as the first line of antioxidant defence (*SOD2* and *CAT*). According to Afifi *et al.* (2017) and Cong *et al.* (2020) changes in the transcription levels of genes encoding antioxidant enzymes can be used as the primary and most sensitive biomarkers of physiological responses to environmental stress. The results showed that zebrafish subjected to HSD and fed 0RJ had lower *SOD2* and *CAT* expression, and, consequently, limited gut antioxidant capacity.

In the Atlantic cod *(Gadus morhua)* short-term stocking HDs enhanced expression of genes related to antibacterial activity, metabolism, and antioxidant defence (Caipang, 2012). Long-term overcrowding, on the other hand, seemed to activate chronic stress responses. Liu *et al.* (2016) observed that juvenile turbot *(Scophthalmus maximus)* bred for 120 days at a HD had lower glutathione, catalase, and superoxide dismutase levels. Dawood *et al.* (2019) found that Nile tilapia *(Oreochromis niloticus)* maintained for 30 days under intensive conditions showed higher lipid oxidation and lower superoxide dismutase, catalase, and glutathione peroxidase activities.

High stocking of unsupplemented zebrafish for 25 days suppressed intestinal antioxidant responses at the transcriptional level, reducing the organ's capacity to eliminate oxidative and toxic substances. Another interesting result observed in this study was that fish reared at NSD and fed 0RJ showed greater *SOD2* and *CAT* expression and antioxidant capacity than animals reared at NSD but fed 10RJ. This finding suggests that, under normal conditions, genes related to the antioxidant are activated, contributing to the control of reactive oxygen species in tissues and cells, thereby maintaining the organism's homeostasis. However, when animals are subjected to an environmental challenge, such as HSD, there may be an increase in the production of reactive oxygen species (Bagni *et al*., 2007; Braun *et al*., 2010), which can damage DNA, RNA, proteins, and lipids (Sayeed *et al*., 2003; Kong & Li, 2010), resulting in transcriptional suppression of these genes and oxidative damage that is often irreversible (Kong & Lin, 2010; Nita & Grzybowski, 2016). Unlike other studies that found an increase in the activity of antioxidant enzymes in animals receiving RJ (Kanbur *et al*., 2009; Silici *et al.*, 2011), we found that dietary supplementation with 10% RJ was not sufficient to maintain and/or increase *SOD2* and *CAT* expression, decreasing the intestinal antioxidant capacity of

animals. This could alter intestinal functions in the short and long terms, adding to the negative effects of environmental stress on the microflora, cellular regenerative capacity, blood flow in the intestinal mucosa, and gastrointestinal motility and secretion (Konturek *et al*., 2011). Wang *et al.* (2018) and Refaey *et al.* (2018) demonstrated that high population densities cause structural and functional changes in fish intestine, negatively influencing food digestion, nutrient absorption, and, ultimately, productive performance.

Conclusions

In summary, the performance of zebrafish reared at HSD decreased as a consequence of intestinal oxidative stress resulting from transcriptional suppression of genes involved in antioxidant responses. These effects culminated in the reduction of the gut's capacity to eliminate toxic substances, affecting intestinal function and reducing nutrient absorption and utilisation. Such a reduction might have contributed to the lower antioxidant capacity of the intestine. The authors concluded that commercial feed alone was not sufficient to maintain the normal functioning of the organism if fish were exposed to environmental challenges. Diet supplementation with 10% RJ seemed insufficient to mitigate the harmful effects of stress on the intestine. The inefficiency of RJ observed in this study might also be related to leaching, as RJ was added to feed by spraying, covering only the surface area of the pellet. To date, this was the first study to evaluate the interaction effects of HSD and RJ supplementation on zebrafish. Further studies are needed for improved understanding of the potential of RJ in fish nutrition.

Acknowledgements

The authors would like to thank the State University of Maringá, the Postgraduate Program in Animal Science and the PeixeGen Laboratory for their technical support. This study was financed with the authors' own resources.

Authors' Contributions

BSP, DABM, FRD, MRS, and ASK wrote the manuscript; RPR, LDC, and EG revised the manuscript; BSP, DABM, FRD, MRS, RPR, LDC, and EG designed the experiment; BSP, DABM, FRD, and MRS conducted animal experiments; BSP, DABM, FRD, MRS, and ASK conducted laboratory analyses; ASK performed the intestinal antioxidant capacity assay; FRD and MRS analysed the data; BSP, DABM, FRD, MRS, RPR and EG secured funding for the project. All authors proofread and approved the final manuscript.

Conflict of Interest Declaration

The authors declare they have no competing interests.

References

Afifi, M., Alkaladi, A., Abu Zinada, O.A. & Couderchet, M., 2017. Alteration in antioxidant genes expression in some fish caught from Jeddah and Yanbu coast as a bio-indicator of oil hydrocarbons pollution. Saudi J. Biol. Sci. 24, 1580-1587. DOI: 10.1016/j.sjbs.2015.06.014

Ahangari, Y.J., Hashemi1, S.R., Akhlaghi, A., Atashi, H., Esmaili, Z., Ghorbani, M., Mastani, R., Azadegan, A. & Davoodi, H., 2013. Effect of *in ovo* injection of royal jelly on post-hatch growth performance and immune response in broiler chickens challenged with Newcastle disease virus. Jour. Appl. Res. 9, 517-529.

Aksakal, E., Ekinci, D. & Supuran, C.T., 2021. Dietary inclusion of royal jelly modulates gene expression and activity of oxidative stress enzymes in zebrafish. Enzyme Inhib. Med. Chem. 36, 885-894. DOI: 10.1080/14756366.2021.1900167

Ashley, P.J., 2007. Fish welfare: Current issues in aquaculture. Appl. Anim. Behav. Sci. 104, 199-235. DOI: 10.1016/j.applanim.2006.09.001

Aslan, Z. & Aksoy, L., 2015. Anti-inflammatory effects of royal jelly on ethylene glycol induced renal inflammation in rats. Int. Braz. J. Urol. 41, 1008-1013. DOI: 10.1590/S1677-5538.IBJU.2014.0470

Babaei, S., Rahimi, S., Karimi Torshizi, M.A. Tahmasebi, G. & Khaleghi Miran, S.N., 2016. Effects of propolis, royal jelly, honey and bee pollen on growth performance and immune system of Japanese quails. Vet. Res. Forum. 7, 13-20.

Bacchetta, C., Rossi1, A.S., Ale, A. & Cazenave, J., 2020. Physiological effects of stocking density on the fish *Piaractus mesopotamicus* fed with red seaweed *(Pyropia columbina)* and β-carotene-supplemented diets. Aquac. Res. 51, 1992-2003. DOI: 10.1111/are.14551

Bagnia, M., Civitareale, C., Priori, A., Ballerini, A., Finoia, M., Brambilla, G. & Marino, G., 2007. Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass *(Dicentrarchus labrax)* and sea bream (*Sparus aurata*). Aquac. 263, 52-60. DOI: 10.1016/j.aquaculture.2006.07.049

Bolasina, S., Tagawa, M., Yamashita, Y. & Tanaka, M., 2006. Effect of stocking density on growth, digestive enzyme activity and cortisol level in larvae and juveniles of Japanese flounder, *Paralichthys olivaceus*. Aquac. 259, 432-443. DOI: 10.1016/j.aquaculture.2006.05.021

Brand-Wiliams, W., Cuvelier, M.E. & Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci. Technol. 28, 25-30. DOI: 10.1016/S0023-6438(95)80008-5

Braun, N., Lima, R.L., Baldisserotto, B., Dafre, A.L. & Nuñer, A.P.O., 2010. Growth, biochemical and physiological responses of *Salminus brasiliensis* with different stocking densities and handling. Aquac. 301, 22-30. DOI: 10.1016/j.aquaculture.2010.01.022

Caipang, C.M.A., 2012. Potential molecular biomarkers of crowding stress in Atlantic cod, *Gadus morhua,* and their importance in health management. Anim. Biol. Anim. Husb. 4, 79-83.

Cong, B., Liu, C., Wang, L. & Chai, Y., 2020. The Impact on antioxidant enzyme activity and related gene expression following adult Zebrafish *(Danio rerio)* exposure to dimethyl phthalate. Animals (Basel). 10, 717. DOI: 10.3390/ani10040717

Costa, Â.A.P., Roubach, R., Dallago, B.S.L., Bueno, G.W., McManus, C. & Bernal, F.E.M., 2017. Influence of stocking density on growth performance and welfare of juvenile tilapia *(Oreochromis niloticus)* in cages. Arq. Bras. Med. Vet. Zootec. 69, 243-251. DOI: 10.1590/1678-4162-8939

Côté, G., Perry, G., Blier, P. & Bernatchez, L., 2007. The influence of gene-environment interactions on GHR and IGF-1 expression and their association with growth in brook charr, *Salvelinus fontinalis* (Mitchill). BMC Genet. 87, 1-13. DOI: 10.1186/1471-2156-8-87

Coutinho, D., Karibasappa, S.N. & Mehta, D.S., 2018. Royal jelly antimicrobial activity against periodontopathic bacteria. J. Int. Dent. 8,18-22. DOI: 10.4103/jid.jid_72_17

Dawood, M.A.O., Shukry, M., Zayed, M.M., Omar, A.A.E-D., Zaineldin, A.I. & Basuini, M.F.E., 2019. Digestive enzymes, immunity and oxidative status of Nile tilapia *(Oreochromis niloticus)* reared in intensive conditions. Slov. Vet.. Zb. 56, 99-108. DOI: 10.26873/SVR-747-2019

Ferreira, D., Rocha, H.C., Kreutz, L.C., Loro, V.L., Marqueze, A., Koakoski, G., Rosa, J.G.S., Gusso, D., Oliveira, T.A., Abreu, M.S. & Barcellos, L.J.G., 2013. Bee products prevent agrichemical-induced oxidative damage in fish. PloS one. 8, 1-11. DOI: 10.1371/journal.pone.0074499

Gonzales, J.M.Jr., 2012. Preliminary evaluation on the effects of feeds on the growth and early reproductive performance of zebrafish *(Danio rerio)*. J. Am. Assoc. Lab. Anim. Sci. 51, 412-417.

Guendouz, M., Haddi, A., Grar, H., Kheroua, O., Saidi, D. & Kaddouri, H., 2017. Preventive effects of royal jelly against anaphylactic response in a murine model of cow's milk allergy. Pharm. Biol. 55, 2145-2152. DOI: 10.1080/13880209.2017.1383487

Hazlerigg, C.R., Lorenzen, K., Thorbek, P., Wheeler, J.R. & Tyler, C.R., 2012. Density-dependent processes in the life history of fishes: Evidence from laboratory populations of zebrafish *Danio rerio*. PLoS One 7, 1-9. DOI: 10.1371/journal.pone.0037550

Herrera, M., Mancera, J.M. & Costas, B., 2019. The use of dietary additives in fish stress mitigation: comparative endocrine and physiological responses. Front. Endocrinol. 10, 1-22. DOI: 10.3389/fendo.2019.00447

Hoseinifar, S.H., Zou, H.K., Van Doan, H., Miandare, H.K. & Hoseini, S.M., 2017. Evaluation of some intestinal cytokines genes expression and serum innate immune parameters in common carp *(Cyprinus carpio)* fed dietary loquat (*Eriobotrya japonica*) leaf extract. Aquac. Res. 49, 120-127. https://doi.org/10.1111/are.13440

Jia, R., Liu, B-L., Han, C., Huang, B. & Lei, J-L., 2016. Influence of stocking density on growth performance, antioxidant status, and physiological response of juvenile turbot, *Scophthalmus maximu*, reared in land-based recirculating aquaculture system. J. World Aquac. Soc. 47, 587-599. DOI: 10.1111/jwas.12295

Kanbur, M., Eraslan, G., Silici, S. & Karabacak, M., 2009. Effects of sodium fluoride exposure on some biochemical parameters in mice: Evaluation of the ameliorative effect of royal jelly applications on these parameters. Food Chem. Toxicol. 47, 1184-1189. DOI: 10.1016/j.fct.2009.02.008

Kong, Q. & Lin, CL.G., 2010. Oxidative damage to RNA: Mechanisms, consequences, and diseases. Cell. Mol. Life Sci. 67, 1817-182. DOI: 10.1007/s00018-010-0277-y

Konturek, P.C., Brzozowski, T. & Konturek, S., 2011. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. J. Physiol. Pharmacol. 62, 591-599.

Liu, B., Jia, R., Han, C., Huang, B. & Lei, J-L., 2016. Effects of stocking density on antioxidant status, metabolism and immune response in juvenile turbot *(Scophthalmus maximus).* Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 190, 1-8, DOI: 10.1016/j.cbpc.2016.07.007

Livak, K.J. & Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. Methods 25, 402-408. DOI: 10.1006/meth.2001.1262

Long, L., Zhang, H., Ni, Q., Liu, H., Wu, F. & Wang, X., 2019. Effects of stocking density on growth, stress, and immune responses of juvenile Chinese sturgeon in a recirculating aquaculture system. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 219, 25-34. DOI: 10.1016/j.cbpc.2019.02.002

Marques, N.R., Hayashi, C., Furuya, W.M. & Soares, C.M., 2004. Influência da densidade de estocagem no cultivo de alevinos de matrinxã *Brycon cephalus* (Günther, 1869) em condições experimentais. Acta Sci. Biol. Sci. 26, 55-59. DOI: 10.4025/actascibiolsci.v26i1.1670

Montero, D., Izquierdo, M.S., Tort, L., Robaina, L. & Vergara, J.M., 1999. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol. Biochem. 20, 53-60. DOI: 10.1023/A:1007719928905

Nagai, T., Inoue, R., Suzuki, N. & Nagashima, T., 2006. Antioxidant properties of enzymatic hydrolysates from royal jelly. J. Med. Food 9, 363-367. DOI: 10.1089/jmf.2006.9.363

NCBI Resource Coordinators, 2018. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 46(D1), D8-D13. doi: 10.1093/nar/gkx1095

Nita, M. & Grzybowski, A., 2016. The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. Oxid. Med. Cell. Longev. 2016, 1-23. DOI: 10.1155/2016/3164734

Peterson, B.C. & Small, B.C., 2005. Effects of exogenous cortisol on the GH/IGF-I/IGFBP network in channel catfish. Domest Anim. Endocrinol. 28, 391-404. DOI: 10.1016/j.domaniend.2005.01.003

Rabbane, M.G., Rahman, M.M., Rahman, M.M., Hossain, M.A. & Hossain, M.A., 2017. Effects of stocking density on growth of zebrafish (*Danio rerio*, Hamilton, 1822). Bangladesh J. Zool. 44, 209-218. DOI: 10.3329/bjz.v44i2.32760

Ramsay, J.M., Feist, G.W., Varga, Z.M., Westerfield, M., Kent, M.L. & Schreck, C.B., 2006. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquac. 258, 565-574. DOI: 10.1016/j.aquaculture.2006.04.020

Refaey, M.M., Li, D., Tian, X., Zhang, Z., Zhang, X., Li, L. & Tang, R., 2018. High stocking density alters growth performance, blood biochemistry, intestinal histology, and muscle quality of channel catfish *Ictalurus punctatus*. Aquac. 492, 73-81. DOI: 10.1016/j.aquaculture.2018.04.003

Sarkar, S., Mukherjee, S., Chattopadhyay, A. & Bhattacharya, S., 2014. Low dose of arsenic trioxide triggers oxidative stress in zebrafish brain: Expression of antioxidant genes. Ecotoxicol. Environ. Saf. 107, 1-8. DOI: 10.1016/j.ecoenv.2014.05.012

Sayeed, I., Parvez, S., Pandey, S., Bin-Hafeez, B., Haque, R. & Raisuddin, S., 2003. Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus* Bloch. Ecotoxicol. Environ. Saf. 56, 295-301. DOI: 10.1016/s0147-6513(03)00009-5

Seven, İ., Şimşek, Ü.G., Gökçe, Z., Seven, P.T., Sur Arslan, A. & Yilmaz, Ö., 2012. The effects of royal jelly on performance and fatty acids profiles of different tissues in quail (*Coturnix coturnix japonica*) reared under high stocking density. Turk. J. Vet. Anim. Sci. 38, 271-277. DOI: 10.3906/vet-1303-62

Seven, P.T., Sur Arslan, A., Özçelik, M., Şimşek, Ü.G. & Seven, İ., 2016. Effects of propolis and royal jelly dietary supplementation on performance, egg characteristics, lipid peroxidation, antioxidant enzyme activity and mineral levels in Japanese quail. Europ. Poult. Sci. 80, 1-16. DOI: 10.1399/eps.2016.138

Silici, S., Ekmekcioglu, O., Kanbur, M. & Deniz, K., 2011. The protective effect of royal jelly against cisplatin-induced renal oxidative stress in rats. World J. Urol. 29, 127-132. DOI: 10.1007/s00345-010-0543-5

Taheri Mirghaed, A., Hoseini, S.M. & Ghelichpour, M., 2018. Effects of dietary 1,8-cineole supplementation on physiological, immunological and antioxidant responses to crowding stress in rainbow trout *(Oncorhynchus mykiss).* Fish Shellfish Immunol. 81, 182-188. DOI: 10.1016/j.fsi.2018.07.027

Van Der Boon, J., Van Den Thillart, G.E.E.J.M. & Addink, A.D.F., 1991. The effects of cortisol administration on intermediary metabolism in teleost fish. Comp. Biochem. Physiol. Part A: Physiol. 1, 47-53. DOI: 10.1016/0300-9629(91)90182-C

Vargesson, N.A., 2007. Zebrafish. In: S.W. Barnett (ed). Manual of animal technology. Blackwell, Oxford, United Kingdom. pp. 78.

Wang, Y-W., Zhu, J., Ge, X-p., Sun, S-M., Su, Y-L., Li, B., Hou, Y-R. & Ren, M-C., 2018. Effects of stocking density on the growth performance, digestive enzyme activities, antioxidant resistance, and intestinal microflora of blunt snout bream (*Megalobrama amblycephala*) juveniles. Aquac. Res. 50, 236-246. https://doi.org/10.1111/are.13889

Wocher, H., Harsányi, A. & Schwarz, F.J., 2011. Husbandry conditions in burbot (*Lota lota L.*): Impact of shelter availability and stocking density on growth and behavior. Aquac. 315, 340-347. DOI: 10.1016/j.aquaculture.2011.01.051