Can Tho University Journal of Science
website: sj.ctu.edu.vn

DOI: 10.22144/ctu.jen.2018.001

Effects of nitrite exposure on haematological parameters and growth in clown knifefish (*Chitala ornata*, Gray 1831)

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Article info.

Received 07 Aug 2017
Revised 06 Nov 2017
Accepted 09 Mar 2018

Keywords

Clown knifefish, growth, haematological parameters, nitrite

ABSTRACT

Physiological responses and growth of clown knifefish (Chitala ornata) (initial weight of 11-12 g) exposed to nitrite were investigated with two separate experiments. The first experiment examined the effects of different nitrite concentrations on haematological parameters. The second experiment examined the effects of different nitrite concentrations on fish growth including 4 treatments such as control, 0.2 mM nitrite, 0.4 mM nitrite, and 4 mM nitrite for measuring growth parameters at days 0, 30, 60, and 90 (sampling 30 fish/tank). There were significant increases in methaemoglobin and leukocytes while other haematological parameters decreased during nitrite exposures at the treatment of 4 mM nitrite. Particularly, methaemoglobin and the number of leukocytes increased from 0.4 to 29.5% and from 39.89x103 to 72.33x103 cells/mm3, respectively. Differently, there were significant declines in the number of erythrocyte (3.19x106–2.33x106 cells/mm3), haemoglobin (10.47-7.04 mM), and haematocrit (38.07-26.5%) at the highest nitrite treatment. After 90 days, daily weight gain (0.25±0.02 g/day), specific growth rate (1.18±0.07 %/day), survival rate (59%) at the treatment of 4 mM nitrite were significantly lower than those of the control, but no significant difference was observed in such parameters between the control and the treatments of 0.2 or 0.4 mM nitrite.

Cited as: Gam, L.T.H., Vu, N.T.T., Nhu, P.N., Phuong, N.T. and Huong, D.T.T., 2018. Effects of nitrite exposure on haematological parameters and growth in clown knifefish (*Chitala ornata*, Gray 1831). Can Tho University Journal of Science. 54(2): 1-8.

1 INTRODUCTION

Air-breathing clown knifefish (*Chitala ornata*, Gray 1831) has high economical value with large-size, fast growth, and high environmental tolerance. This species which plays an important role in domestic need and exportation has been popularly cultured in Hau Giang province, Vietnam (Nguyen Thanh Long, 2015). The total aquaculture production was 43,909 tons in 2009 and 44,429 tons in 2011 (DARD, 2011). The intensive culture of clown knifefish in pond and cage has recently been expanded to other freshwater locations of the Mekong Delta, Vietnam.

The intensive culture of fish in pond usually causes poor water quality from oxygen deficiency and high amount of toxic compounds such as carbon dioxide, hydro-sulfur, nitrite, and nitrate. Also, the overfeeding and excessive using of fertilizers cause accumulation of organic matters, and the decomposition of these organic compounds produces toxins such as ammonia (NH_3^+) and nitrite (NO_2^-) (Le Van Cat *et al.*, 2006). Nitrification and denitrification processes of bacteria in aquatic system produce nitrite (NO_2^-), subsequently leading the high loading of organic matters, and nitrogenous products (Eddy and Williams, 1987; Hargreaves, 1998;

Jensen, 2003). However, the elevation of water nitrite level causes multiple physiological disturbances in freshwater systems such as ion regulatory, excretory, endocrine, respiratory, and cardiovascular processes through the active nitrite uptake across the gills (Naylor *et al*., 2000; Kroupova *et al.,* 2005; Svobodová *et al.,* 2005). Nitrite is toxic to aquatic animals and considered to be an oxidising product (Lewis and Morris, 1986). Although nitrite normally accumulates in the water below 1 µM (Jensen, 2003), nitrite levels in fish body fluids can reach higher concentration compared to environmental nitrite levels due to the competition between nitrite and chloride with the same uptake mechanism for Cl^-/HCO_3^- exchange in fish gills (Eddy and Williams, 1987; Jensen, 2003). The air-breathing fish species can have higher tolerance of nitrite compared to water-breathing fish such as a 96-h LC_{50} for nitrite of 1.65 mM in the facultative air-breather, striped catfish *(Pangasionodon hypophthalmus*) (Lefevre *et al*., 2011), and 4.9 mM in the obligate air-breathing, snakehead *(Channa striata)* (Lefevre *et al*., 2012) with unusual responses after 24 to 72-h in nitrite exposure. Typically, the air-breathing clown knifefish have become the most nitrite tolerance species with 96-h LC_{50} of 7.82 mM (Gam *et al.,* 2017). Therefore, the effects of nitrite on haematological parameters and growth in clown knifefish were investigated to provide an understanding about physiological responses of another air-breathing fish species in intensive culture system in the Mekong Delta.

2 MATERIALS AND METHODS

The clown knifefish juveniles from the hatcheries were transported to the wet laboratories in Can Tho University and acclimated in a 1 m^3 tanks in 2 weeks at 27±1 ºC and constant aeration. The water (30%) was changed every two days to control optimal environmental conditions (Boyd, 1990). A mixture of commercial feed and annelid worms was used as fish feed during acclimation and experiment (Shrimp commercial feed with 38% protein, Tomboy Aquafeed Company, Vietnam). Nitrite used during experimentation was $NaNO_2$ (Merck, Germany). The experiments were investigated based on Vietnamese national guidelines for animal welfare.

2.1 Effects of nitrite on haematological parameters in clown knifefish (*C. ornata)*

Fish (initial weight of 11.93±0.81 g, n=800) were randomly collected from 1 m^3 holding tanks with optimal water quality, and subsequently distributed to 16 500-L tanks (200-L water and 50 fish per tank). The water in these tanks was continuously aerated in two days prior experimentation. From the nitrite tolerance of clown knifefish (96-h LC_{50} of 7.82 mM nitrite, Gam *et al.,* 2017), the physiological experiment included 4 treatments such as 0 mM (0 mg/L,control), 0.2 mM (9.2 mg/L, recommended concentration), 0.4 mM (18.4 mg/L, 5% of 96-h LC_{50}), and 4 mM nitrite (184 mg/L, 50% of 96-h LC_{50}), with 4 replicates (4 tanks) for each treatment. These concentrations of NO_2^- were calculated from dissociated equation of $NaNO_2^-$ below:

$$NaNO_2 \rightarrow Na^+ + NO_2^-$$
$$69\ g \qquad 46\ g$$
$$Y\ (g) \qquad X\ (g)$$

Therefore, Y ($NaNO_2$ used) = (69 x X)/46 (g)

Nitrite in the water was recorded twice a day, and extra nitrite was added to maintain desirable concentrations during experimentation by spectrophotometer using the Griess reaction (Lefervre *et al.,* 2011, 2012). Three fish were sampled from each tank at days 0, 1, 3, 7, and 14 during experiment of 2 weeks. The ice was used for a comatose situation in fish before sampling blood. A total volume of 300 µL of blood was collected from the caudal vein of each fish by a heparinised syringe for measuring haematological parameters including the number of erythrocytes and leukocytes, haematocrit (ratio between volume of red blood cells), mean corpuscular haemoglobin concentration (MCHC) (Do Thi Thanh Huong and Nguyen Van Tu, 2010), haemoglobin and methaemoglobin (Jensen, 2007).

2.2 Effects of nitrite on growth of clown knifefish (*C. ornata)*

Fish (initial weight 11.53±0.15 g, n=600) were randomly taken from 1 m^3 holding tanks with optimal water quality and subsequently distributed to 12 500-L tanks (300-L water and 50 fish per tank) with aerated water two days before experimentation. The experiment included 4 treatments such as 0 mM (control), 0.2 mM, 0.4 mM, and 4 mM nitrite, with 3 replicates (3 tanks) for each treatment in 90 culturing days. Nitrite in the water was recorded every three days before exchanging water (30%), and subsequently extra nitrite was added for maintaining the chosen concentrations. The fish were fed with commercial pellets with feeding rate of 5% of body weight. Humidity of commercial pellets (Shrimp feed with 38% protein, Tomboy Aquafeed Company, Vietnam) was less than 10%. The pellets had uniform size (1 g = 203 pellets). Uneaten feed after 30 minutes of feeding was calculated for determination of feed used. Thirty fish per tank were sampled on the days 0, 30, 60 and 90

for measuring growth parameters including weight gain (WG), daily growth rate (DWG), specific growth rate (SGR), FCR (feed conversion ratio), and survival rate (SR). They were calculated as follows:

$$WG = W_t - W_0$$

$$DWG = (Wt - W_o)/t$$

$$SGR\ (\%/day) = [(LnWt - LnW_0)]/t)x100$$

Where, W_0: Initial weight of fish (g); W_t: Final weight of fish (g); t: rearing time (day)

FCR = (feed used)/(total weight of fish harvested– total weight of fish stocked) + (total weight of dead fish).

SR (%) = 100x(total fish harvested/total fish stocked)

2.3 Data analysis

All figures were made in sigma plot 12.5. All data were analyzed with PASW statistics (SPSS 18.0). Predicted mean, upper and lower 95% confidence intervals for the 96-h LC_{50} were analyzed in JMP 9.0 using a logistic model. A two-way ANOVA (the Holm-Sidak multiple comparison method, pair-wise comparison) was used to identify differences between treatments and sampling times for all haematological parameters. A one-way ANOVA was used to identify differences between treatments for growth parameters. A p value of less than 5% (p<0.05) was judged significant. All data are shown as standard error of the mean (SEM).

3 RESULTS AND DISCUSSION

3.1 Effects of nitrite on haematological paramters in clown knifefish *C. ornata*

There was no significant difference in erythrocytes between sampling times with the value from 3.04±0.23 to 3.27±0.29x10^6 cells/mm^3 in control treatment as well as those at the treatment of 0.2 mM nitrite, while the number of erythrocytes had significant decreasing trends in higher nitrite concentration treatments. The number of erythrocyte dropped to the lowest values at the treatments of 0.4 mM and 4 mM nitrite (2.76±0.36 and 2.33±0.53x10^6 cells/mm^3, respectively). However, there were a significant recovery in the number of erythrocyte at these two treatments at experimental termination (2.87±0.41 and 2.71±0.50x10^6 cells/mm^3, respectively) compared to controls (3.10±0.19x10^6 cells/mm^3) (p<0.05) (Table 1). Blood cell responses are important indicators of changes in the internal and/or external environment of animals. Exposure to chemical pollutants in fish can induce both increases and decreases in haematological levels. Their changes depend on fish species, age, the cycle of the sexual maturity of spawners and diseases (Luskova, 1997). In contrast, the number of leukocytes reached the highest values at day 3 (52.16±3.37, 55.14±3.26, and 67.32±4.27x10^3 cells/mm^3 at the treatments of 0.2, 0.4 and 4 mM nitrite, respectively), and it maintained significantly higher than those at the controls (39.89±2.91 to 41.74±3.53x10^3 cells/mm^3) with the values (44.73±4.65, 46.37±3.94 and 57.84±3.10x10^3 cells/mm^3, respectively) at day 14 (p<0.05) (Table 1). According to Das *et al.* (2004a), the number of erythrocytes of *Cirrhinus mrigala* decreased significantly after 6 h in nitrite exposures of 8 mg/L and 10 mg/L, and the number of erythrocytes of *Catla catla* decreased at 6 h, recovered at 12 h, and then decreased again at 96 h by 21.2-31.8% in nitrite exposures of 1.0-10.4 mg/L nitrite (Das *et al*., 2004b).

Table 1: Number of erythrocytes and leukocytes after 14 days exposed to nitrite

Treatment	Day 0	Day 1	Day 3	Day 7	Day 14
Number of erythrocytes					
Control	3.10±0.19	3.13±0.31	3.19±0.50	3.05±0.29	3.17±0.32
0.2 mM	3.11±0.25	2.88±0.36	2.82±0.31^{+}	2.92±0.42	2.93±0.57
0.4 mM	3.03±0.38	2.81±0.45	2.76±0.36$^{*,+}$	2.85±0.47	2.87±0.41
4 mM	3.04±0.34	2.55±0.40$^{*,+}$	2.33±0.53$^{*,+}$	2.53±0.76$^{*,+}$	2.71±0.50$^{*,+}$
Number of leukocytes					
Control	40.47±3.70	40.70±3.58	41.43±4.10	40.61±3.47	41.04±3.74
0.2 mM	41.26±3.33	45.86±3.63$^{*,+}$	52.16±3.37$^{*,+}$	46.42±4.36$^{*,+}$	44.73±4.65$^{*,+}$
0.4 mM	41.29±3.43	47.27±4.01$^{*,+}$	55.14±3.26$^{*,+}$	48.78±3.14$^{*,+}$	46.37±3.94$^{*,+}$
4 mM	40.41±2.85	58.57±3.96$^{*,+}$	67.32±4.27$^{*,+}$	64.81±4.85$^{*,+}$	57.84±3.10$^{*,+}$

Notes: Asterisks show significant differences from day 1, day 3, day 7, and day 14 compared to day 0 in the same treatment and plus signs show significant differences from groups of 0.2 mM, 0.4 mM and 4 mM nitrite compared to the control group on a given day. Showed data are mean ± standard error (n=12)

Nitrite enters the membrane of red blood cells, subsequently reacted with haemoglobin (Hb), causing oxidation of the haem iron (from Fe^{2+} to Fe^{3+}) for methaemoglobin (metHb) and nitrate formations, leading the reduction in Hb concentration (Kosaka and Tyuma, 1987; Jensen, 2009; Jensen and Rohde, 2010). Most fish species appear an elevation in metHb (a form of oxidised Hb in higher levels of nitrite exposure (Brauner *et al*., 1993; Paula-Silva *et al.,* 1996; Duncan *et al*., 1999). And metHb cannot bind with oxygen, thus causing oxygen transferring impairment from blood to tissues (Jensen, 1990). Following Margiocco *et al.* (1983), metHb in rainbow trout *(Salmo gairdnery)* reached 41.83% after 24 h exposed to 0.68 mg/L nitrite. MetHb in milkfish increased to 68.7% after 12 h exposed to 14 mg/L nitrite (Almendras, 1987). In facultative air-breathing fish *P. hypophthalmus*, metHb increased to 63% of Hb on day 1, and decreased to 28% of Hb on day 7 in nitrite exposure of 0.9 mM (Lefevre *et al*., 2011). Similarly, in snakehead *(Channas striata)*, the percentage of metHb increased to 30% at day 2, but decreased by 5% of total Hb on day 7 (Lefevre *et al*., 2012).

Similarly, in this study, metHb significantly increased and reached the highest percentages on day 3 (2.55±0.12, 4.30±0.32, and 29.54±0.72% at the treatments of 0.2, 0.4 and 4 mM nitrite, respectively). Nevertheless, there was a reduction in metHb at experimental termination with the values (1.40±0.15, 2.53±0.21 and 18.38±0.95 % at these three treatments, respectively), which were significantly different from controls (below 1%) ($p<0.05$) (Figure 1A). This result was similar to the recent study of Gam *et al.* 2017, whereas metHb in clown knifefish reached 38% at day 2 and then reduced to 17% at day 7 in nitrite exposure of 2.5 mM. The decreases in metHb were accompanied with denitrification process converting nitrite to nitrate which is considered non-toxic (Camargo *et al.,* 2005; Gam *et al.,* 2017). In addition, the metHb reduction was explained by up-regulation of metHb reductase activity converting metHb to functional Hb, particularly the first order rate constant for metHb reduction by erythrocyte metHb reductase rose from 0.01 in control group to 0.046 min-1 in 2.5 mM nitrite after 6 days in the air-breathing clown knifefish (Gam *et al.,* 2017). Some previous studies showed an up-regulation of metHb reductase, typically the water-breathing carp (Knudsen and Jensen, 1997).

Hb concentration is converted to metHb and loses capacity with oxygen. Higher concentrations of nitrite exposure cause higher concentrations of metHb generated and lower concentration of Hb (Kosaka and Tyuma, 1987; Jensen, 2009; Do Thi Thanh Huong and Nguyen Van Tu, 2010). Although there was a slight recovery after a sharp drop on day 3 (8.48±0.95 and 7.11±0.66 mM of the treatments 2 and 4 mM nitrite), Hb concentration on day 14 maintained significantly lower compared to that of control (10.23±1.05 to 10.66±0.93 mM) with the values of 9.31±1.46 and 7.59±1.37 mM at these two nitrite treatments, respectively ($p<0.05$) (Figure 1B). Haematocrit had a decreasing tendency during nitrite exposures; particularly it significantly felt to 27.62±4.22% on day 3 at the highest level of nitrite exposure. However, there was a modest increase (31.75±3.75), which was significantly lower than that at controls (38.07±4.58 to 40.48±4.08%) of this treatment at experimental termination (Figure 1C). Mean corpuscular haemoglobin concentration (MCHC) generally decreased slightly (26.55±4.44 - 24.29±5.60 mM) during nitrite exposures (Figure 1D); but there was no significant difference on this parameter among all treatments.

MetHb formation is related to the formation of free peroxide and changes the properties of essential protein, including Hb and composition of erythrocyte membrane causing the reduction in Hb solubility, which damage erythrocyte structures and decompose them rapidly (Everse and Hsia, 1997; Bloom and Brandt, 2001). Nitrite reduces total Hb in several species such as common carp *(Cyprinus carpio)* (Jensen *et al.,* 1987), the red-tailed Brycon (Avilez *et al*., 2004), rainbow trout *(Oncorhynchus mykiss)* (Stormer *et al*., 1996; Aggergaard and Jensen, 2001), whereas it causes the decrease in haematocrit in the air-breather *Hoplosterum littorale* (Duncan *et al.*, 1999). Haematocrit in striped catfish *P. hypophthalmus* was significantly decreased after 1 and 2 days in exposures to 0.9 mM nitrite compared to day 0; but it returned to control values by day 4 and 7 (Lefevre *et al.*, 2011). Differently, haematocrit, Hb and MCHC in snakehead *C. striata* had a slight rise during exposures of 1.4 and 4.0 mM nitrite in 7 days, but there was generally no significant effect of nitrite on these haematological parameters (Lefevre *et al.,* 2012).

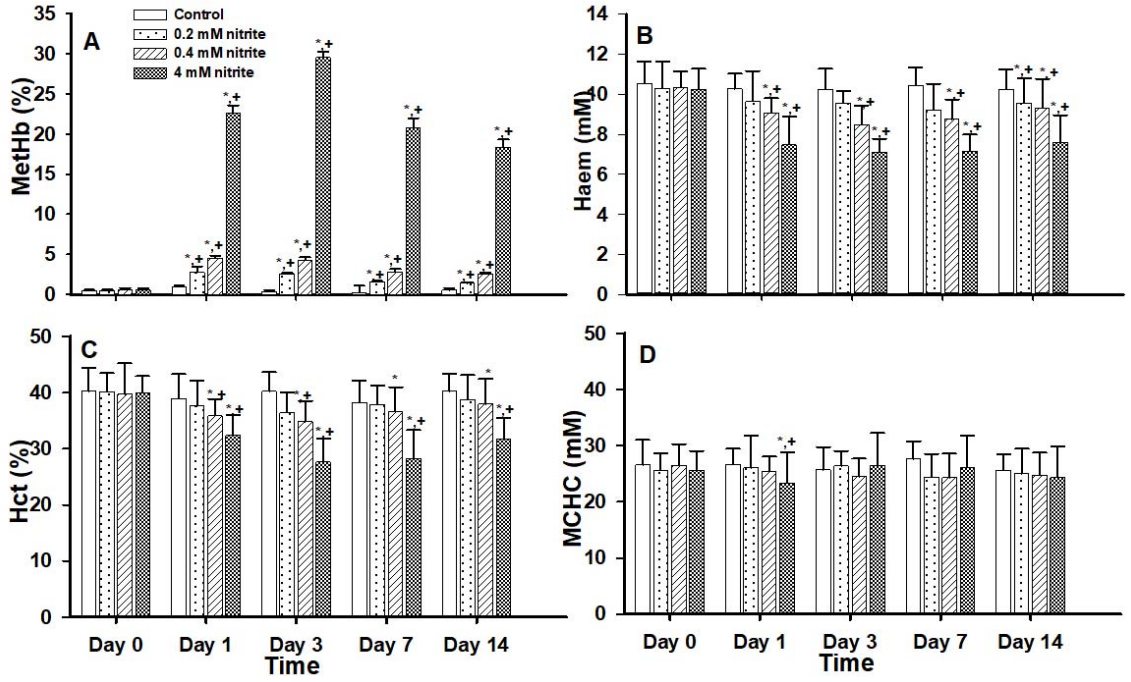

Fig. 1: Haematological paramters in *C. ornata* after 14 days exposed to nitrite (control, 0.2 mM, 0.4 mM, and 4 mM. (A) Methaemoglobin (metHb), (B) Haemoglobin concentration, (C) Haematocrit (Hct), and (D) mean corpuscular haemoglobin concentration (MCHC)

Asterisks show significant differences from day 0 in the same treatment and plus signs show significant differences from the control group on a given day. Showed data are mean ± standard error (n=12)

3.2 Effects of nitrite on growth parameters in clown knifefish *C. ornata*

After 90 days of culturing, there was no significant difference in weight gain (WG) of the control and 0.2 mM nitrite treatments. However, weight gain of treatments of 0.4 mM and 4 mM nitrite (31.12±2.01 and 22.54±1.23 g, respectively) was significantly different in comparison with control (35.85±1.93 g) at experimental termination (p<0.05) (Table 2). The accumulation of organic matters causes the formation of microbial metabolites, such as ammonia, nitrite, and hydrogen sulfide into the water column, leading to chronic stress on fish during the culture (Das *et al.*, 2004a). The formed stress may subsequently cause exhaustion, diseases, and mortality in fish (Francis-Floyd, 1990). This result was similar to the study on the growth performance of silver carp *(Puntius gonionotus)* expose to nitrite. The growth rate of silver carp was significantly decreased at the treatment of 2 mg/L nitrite compared to that of control. This study also addressed that survival rate of fish was significantly higher than that of the treatment of 4 mg/L (p<0.05) (Yusoff *et al*., 1998). Following to Colt *et al*. (1981), the growth rate of channel catfish (*Ictalurus punctatus)* was significantly reduced after 31 culturing days in nitrite exposure of 1.60 mg/L, and the mortality significantly increased in nitrite treatment of 3.71 mg/L. Similarly, the study of Do Thi Thanh Huong and Le Tran Tuong Vi (2013) documented that the growth rate of snakehead *C. striata* was significantly decreased after 90 days exposed to the nitrite at concentrations of 184.6 mg/L and 201.6 mg/L compared to that of control and treatment of 11.94 mg/L nitrite.

The treatment of 4 mM nitrite after a 90-day culture had the lowest specific growth rate (SGR) (1.18±0.07 %/day), which was significantly different from that of the control, 0.2 mM and 0.4 mM nitrite treatments (1.56±0.04, 1.52±0.06, 1.45±0.07, respectively) (Table 2). Meanwhile, there was no significant difference in DWG between control and 0.2 mM nitrite treatment while that of the two treatments of 0.4 mM and 4 mM nitrite (0.35±0.03, 0.25±0.02 g/day) was significantly lower compared to control (0.40±0.02 g/day) (p<0.05) (Table 2). The experimental result showed that the growth rate was limited during chronic nitrite exposures. This may be resulted from the metHb and HbNO formation, causing the low of oxygen capacity in the blood, subsequently affecting the fish growth during chronic nitrite exposures (Jensen, 2007).

Table 2: Initial weight (W_0), weight at day 90 (W_{90}), WG, SGR, and DWG after 90 days exposed to nitrite

Treatment	Parameters				
	W_0	W_{90}	WG	SGR (%/day)	DWG (g/day)
Control	11.67±0.15^a	47.52±2.02^a	35.85±1.93^a	1.56±0.04^a	0.40±0.02^a
0.2 mM	11..53±0.15^a	45.49±2.32^{ab}	33.96±2.18^{ab}	1.52±0.06^a	0.38±0.03^{ab}
0.4 mM	11.53±0.21^a	42.65±1.71^b	31.12±2.01^b	1.45±0.07^a	0.35±0.03^b
4 mM	11.83±0.31^a	34.37±1.36^c	22.54±1.23^c	1.18±0.07^b	0.25±0.02^c

Notes: Showed data were mean ± standard error. The values at the same column with same letters were insignificantly different (p> 0.05)

The higher concentrations of nitrite were accompanied with the lower survival rate in all nitrite treatments. Typically, the treatment of 4 mM nitrite (59%) had the lowest survival rate, which was significantly different from the treatments of control, 0.2 and 0.4 mM nitrite (95, 92 and 86%) (Figure 2A). FCR gradually increased from low to high nitrite levels being exposed. Two treatments of 0.4 mM and 4 mM nitrite reached the highest FCR values (4.19 ± 0.08 and 4.56 ± 0.11, respectively). These values were significantly different from control (3.88±0.12) (p<0.05) (Figure 2B). The feeding efficiency and survival rate at the control treatment was highest among all treatments while the highest amount of feed used was accompanied with the lowest survival rate at the treatment of 4 mM. At the treatments of low nitrite concentrations (0.2 and 0.4 mM nitrite), survival rate decreased, but maintained insignificantly different from the control. A possible explanation is that fish can adapt with nitrite environment and increases their nitrite tolerance via denitrification process converting nitrite to nitrate and up-regulation of metHb reductase enzyme converting metHb to Hb (Huey and Beitinger, 1982; Mohr *et al*., 1986). These results were similar to the study of Colt *et al.* (1981) showing the decrease of survival rate in nitrite exposure of 3.71 mg/L. Similar results were obtained by Do Thi Thanh Huong and Le Tran Tuong Vi (2013) on snakehead, *C. striata* with FCR (1.79±0.1) in nitrite exposure of 201.6 mg/L, while the treatment of 11.94 mg/L nitrite had lower FCR (1.19±0.05), which was not significantly different compared to that of the control treatment (1.38±0.27).

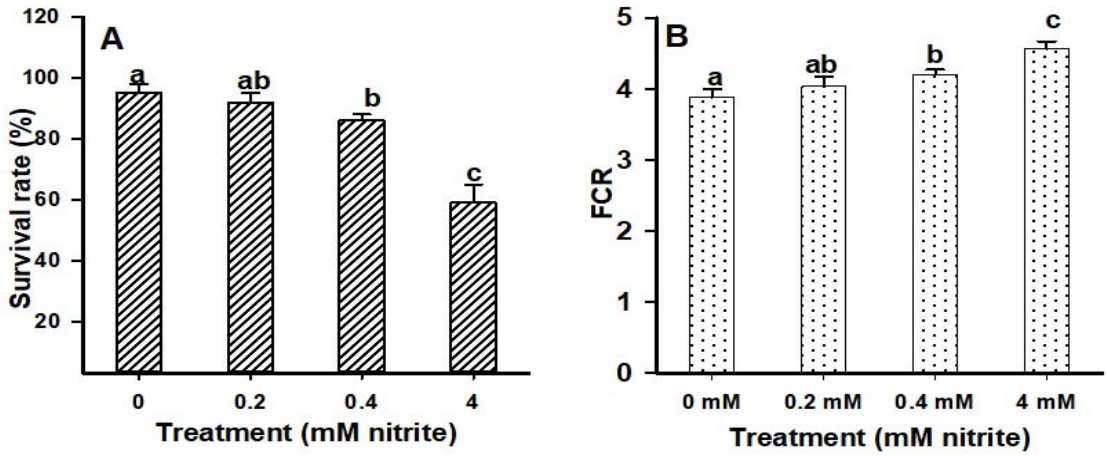

Fig. 2: Growth paramters in *C. ornata* after 90 days exposed to nitrite (control, 0.2 mM, 0.4 mM and 4 mM). (A) Survival rate (SR) and (B) Feed conversion ratio (FCR)

Showed data were mean ± standard error. The values at the same column with same letters were insignificantly different (p>0.05)

4 CONCLUSIONS

One of the most nitrite tolerant air-breathing clown knifefish had significant effects on the number of haematological cells, metHb, haematocrit and Hb concentration, while there was no remarkable change of mean corpuscular Hb concentration after 14 days exposed to nitrite. Growth parameters had a significant difference during nitrite exposures; growth rate and survival rate were significantly low of the treatments 0.4 mM and 4 mM nitrite. In addition, nitrite caused the lower efficiency of feed used at the highest nitrite concentrations (0.4 mM and 4 mM nitrite) with the highest FCR values (4.19 ± 0.08 and 4.56 ± 0.11) compared to control value.

ACKNOWLEDGEMENTS

The research was conducted at College of Aquaculture and Fisheries, Can Tho University (Vietnam), and funded by Inter-disciplinary Project on Climate change in Tropical Aquaculture (iAQUA), the Danish Ministry of Foreign Affairs (DANIDA) [DFC no.12-014AU]. Special thanks are given to Assoc. Prof. Dr. Mark Bayley, Prof. Dr. Tobias Wang and Assoc. Prof. Dr. Frank Bo Jensen for their valuable comments.

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