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Oxidative stress parameters in silver catfish (*Rhamdia quelen*) juveniles infected with *Ichthyophthirius multifiliis* and maintained at different levels of water pH

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ABSTRACT

The aim of this study was to determine oxidative stress parameters in the liver, gill and muscle of silver catfish juveniles infected with *Ichthyophthirius multifiliis* and maintained at pH 5.0 or 7.0 for three days. Juveniles were infected by adding one *I. multifiliis*-infected juvenile and water containing theronts to tanks. After the appearance of white spots on the skin, infected juveniles exposed to pH 5.0 and 7.0 showed significantly higher thiobarbituric acid reactive substances (TBARS) levels in the liver and gills compared to uninfected juveniles. Liver of infected juveniles exposed to pH 7.0 showed higher catalase (CAT) and lower glutathione-S-transferase (GST) activities, but those maintained at pH 5.0 showed significantly higher GST activity than uninfected juveniles. The gills of infected juveniles showed significantly higher CAT (day two) and GST activity at both pH 5.0 and 7.0 compared to uninfected juveniles. Muscle of infected juveniles showed significantly lower CAT and GST activity and TBARS levels (at day three) when maintained at both pH 5.0 and 7.0 compared to uninfected juveniles. In conclusion, *I. multifiliis* infection induces liver and gill damage via lipid peroxidation products in silver catfish, but higher antioxidant enzyme activity could indicate a greater degree of protection against this parasite.

1. Introduction

The silver catfish *Rhamdia quelen* (Quoy and Gaimard) is an endemic fish species of South America and is the primary native species raised in southern Brazil (Gomes et al., 2000; Baldisserotto, 2009). Juveniles of this species tolerate a pH range of 4.0–9.5 without mortality (Zaions and Baldisserotto, 2000), but the optimal pH is 7.5 (Copatti et al., 2005). One of the main problems in the production of silver catfish is ichthyophthiriasis, also known as white spot disease (Miron et al., 2003; Garcia et al., 2007), which is caused by infection with the protozoan ciliate *Ichthyophthirius multifiliis* (Matthews, 2005). This protozoan is an endoparasite that causes economic loss in worldwide commercial and ornamental fish culture (Miron et al., 2003; Matthews, 2005; Garcia et al., 2007), and the intensity of its infection in silver catfish varies with water pH (Garcia et al., in press). The trophont stage (cyst tissue in the host) causes damage to the host epidermis and gill epithelium, compromising gas exchange (Ewing et al., 1994), which could lead to oxidative stress.

Oxidative stress is defined as an unbalanced state between pro-oxidants and antioxidants, resulting in elevated production of reactive oxygen species (ROS) and free

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radicals, agents with the potential to produce deleterious effects (Matés et al., 1999). Parasitic infections may induce alterations in the oxidative status and/or antioxidant defenses of parasitized fish (Martinez-Álvarez et al., 2005). Fish infected with *I. multifiliis* present localized immune responses characterized by epidermal infiltration by granulocytes and neutrophils. Ichthyophthiriasis also induces general effects in fish hosts, leading to significant changes in physiological and biochemical characteristics (Matthews, 2005). Inflammatory processes initiated by macrophages and neutrophils cause the production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which initiate a series of events that increase the formation of ROS and free radicals (Jones et al., 2000), which can increase damage to host tissues.

Therefore, the aim of this study was to analyze oxidative stress parameters in the liver, gill and muscle tissues of silver catfish juveniles infected with *I. multifiliis* and maintained at different pHs.

2. Materials and methods

2.1. Experimental animals

Silver catfish juveniles (32.5 ± 5.5 cm and 72.1 ± 11.0 g) were obtained from a fish farm near Santa Maria city, southern Brazil. These juveniles did not present any apparent signs of diseases when they arrived at the Fish Physiology Laboratory at the Universidade Federal de Santa Maria, where they were maintained for seven days in six continuously aerated (using two 20 W air pumps) 250-L tanks. Water quality in this tanks were (mean ± SEM): pH 7.5 ± 0.1, hardness 24.0 ± 0.5 mg/L $CaCO_3$, water alkalinity 17.8 ± 0.3 mg/L $CaCO_3$, temperature $24.1 \pm 2\,^{\circ}C$, dissolved oxygen levels 6.4 ± 0.8 mg/L, maximum total ammonia levels 0.35 ± 0.02 mg/L, maximum un-ionized ammonia levels 0.03 ± 0.01 mg/L, maximum nitrite levels 0.05 ± 0.01 mg/L.

2.2. Juvenile infection and intensity of trophonts

Forty juveniles were infected by adding one silver catfish juvenile infected with *I. multifiliis* (more than 1000 trophonts) and by 1 L of water infected with approximately 1000 theronts (free form) to each 250-L tank, simulating the infection condition in a fish culture. Juveniles maintained in these tanks began to present white spots after seven days (80–120 trophonts/fish). They were then transferred to four continuously aerated 250-L polypropylene tanks at pH 5 and 7, two replicates each, with ten infected juveniles (80–120 trophonts/fish) maintained in each replicate for three days. These pH values were chosen because the intensity of *I. multifiliis* infection is lower in silver catfish maintained at pH 5.0 compared to those kept at pH 7.0 (Garcia et al., in press). A semi-static system was used and 10% of the water volume was changed daily. The same experimental procedures (except infection) were performed with uninfected juveniles (control group).

The intensity of trophonts was determined by counting white spots on the skin and gills (each white spot was considered as a parasite) (Bush et al., 1997) of one anesthetized fingerling (40 mg/L clove oil) per replicate with the assistance of a stereomicroscope (total magnification 10×).

2.3. Tissue collection

Three specimens were collected from each replicate of both uninfected and infected fish at 1, 2 and 3 days after the appearance of white spots. These fish were killed by spinal column section and tissues (gills, liver and muscle) were removed, weighed separately and immediately frozen in liquid argon. The tissues were then stored in a $-70\,^{\circ}C$ freezer for subsequent analysis.

2.4. Water quality

Water pH was measured twice a day with a DMPH-2 (Digimed, São Paulo, Brazil) pH meter and adjusted according to the experimental conditions (pH 5.0 and 7.0). Total ammonia levels were determined twice a week by nesslerization according to the method of Greenberg et al. (1976). Un-ionized ammonia levels were calculated according to the method of Piper et al. (1982). Dissolved oxygen and temperature were measured daily with an YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA) and laboratory temperature was maintained by using an air conditioner. Total alkalinity, nitrite (Boyd, 1998), and water hardness levels (Greenberg et al., 1976) were determined once.

Water pH in tanks was changed to the experimental pH by adding 0.1 M H_2SO_4 to tanks containing fish. In all treatments, juveniles where fed once a day (08:00) with a commercial diet (Supra, 32% CP, Alisul Alimentos, Carazinho, Brazil) at 5.0% of their body mass. Uneaten food as well as other residues and feces were siphoned out 30 min after feeding.

2.5. Parameters of oxidative stress

Liver, gill and muscle tissues were homogenized in 1.15% (w/v) KCl solution containing 1 mM phenylmethylsulphonyl-fluoride (PMSF). Homogenates were centrifuged at 600 *g* for 10 min to eliminate nuclei and cell debris and the supernatant fraction obtained was frozen at $-70\,^{\circ}C$ for further measurements. Supernatants were used for analysis of thiobarbituric acid reactive substances (TBARS), catalase (CAT) and glutathione-S-transferase (GST). Lipid peroxidation was measured by TBARS using methods described by Buege and Aust (1978). Briefly, absorbance measurements at 535 nm were used to measure the reaction between thiobarbituric acid and lipoperoxidation (LPO) products, resulting in the formation of a chromogen (Schiff's base). Results were reported as nmol/mg protein. The protein content of homogenates was measured using methods described in Lowry et al. (1951), using bovine serum albumin as the standard. Catalase activity was determined by according to methods described by Boveris and Chance (1973), in which H_2O_2 loss is followed spectrophotometrically at 240 nm. Results were reported as pmol/mg protein. GST activity was determined spectrophotometrically at 340 nm using the method described in Habig et al. (1974). Activity

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Table 1
Physicochemical parameters of the water in the experimental tanks.

Physicochemical parameters	Experimental pH	
	5.0 (5.2 ± 0.4)	7.0 (6.7 ± 0.5)
Dissolved oxygen (mg/L)	6.4 ± 0.6	6.5 ± 0.2
Temperature (°C)	24.1 ± 2.0	23.5 ± 1.0
Hardness (mg $CaCO_3$/L)	18.0 ± 6.0	20.0 ± 4.0
Alkalinity (mg $CaCO_3$/L)	7.6 ± 2.0	32.0 ± 2
Total ammonia (mg/L)	0.4 ± 0.01	0.3 ± 0.01
Non-ionized ammonia (mg/L)	0.04 ± 0.01	0.03 ± 0.01
Nitrite (mg/L)	0.05 ± 0.02	0.05 ± 0.02

was calculated by monitoring changes in absorbance at 340 nm using an extinction coefficient of 9.6 mmol/cm. One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1 μmol of CDNB (1-chloro-2,4-dinitrobenzene) with GSH per minute at 25 °C.

2.6. Statistical analysis

Data are reported as means ± SEM (N). Homogeneity of variances among groups was tested with the Levene test. Data for TBARS, CAT and GST had homogeneous variances and comparisons between different treatments were made using two-way analysis of variance and the Dunnet test. Analysis was performed using Statistica software (version 5.1) and minimum significance levels were set at $P < 0.05$.

3. Results

The physicochemical parameters of the water used in our experiments did not show any significant difference between treatments (except alkalinity, due to pH) (Table 1). Oxidative stress parameters from uninfected fish did not show any significant differences as a function of the time of collection and pH (Figs. 1–3).

Silver catfish juveniles infected with *I. multifiliis* (three days after the appearance of white spots on the skin) and exposed to pH 5.0 and 7.0 showed significantly higher TBARS levels in the liver (988 and 176%, respectively) than uninfected juveniles (Fig. 1A and B). CAT activity in the liver of silver catfish exposed to pH 7.0 was significantly lower (57, 50 and 61%, days 1, 2 and 3, respectively) in infected individuals than in uninfected juveniles (Fig. 1D). Livers of infected silver catfish juveniles showed significantly higher GST activity at all time points at both pH 5.0 (1229, 823 and 732%) and 7.0 (1372, 835 and 2165%) compared to uninfected juveniles (Fig. 1E and F).

Infected juveniles kept at pH 5.0 showed significantly higher TBARS levels in the gills at days two and three (238 and 243%, respectively), while those maintained at pH 7.0 showed significantly higher TBARS levels at day three (216%) compared to uninfected juveniles (Fig. 2A and B). CAT activity in the gills of infected silver catfish juveniles exposed to pH 5.0 and 7.0 was significantly higher at day two (339 and 327%, respectively), but was significantly lower at day three (39 and 11%, respectively) compared to uninfected fish (Fig. 2C and D). The gills of infected juveniles showed significantly higher GST activity at all time points when maintained at pH 5.0 (350, 464 and 390%) and 7.0 (278, 495 and 213%) compared to uninfected juveniles (Fig. 2E and F).

Muscle tissues of infected silver catfish juveniles maintained at pH 5.0 showed significantly lower TBARS levels (44%) at day three compared to uninfected juveniles (Fig. 3A). CAT activity was also significantly lower in the muscles of infected juveniles maintained at pH 5.0 and 7.0 at all time points except day 1 at pH 7.0 (59, 75 and 41%; 50 and 57%, respectively) compared to uninfected juveniles (Fig. 3C and D). Muscle tissues of infected juveniles showed significantly lower GST activity at all time points when maintained at both pH 5.0 (25, 44 and 19%) and 7.0 (32, 43 and 73%) compared to uninfected juveniles (Fig. 3E and F).

4. Discussion

Infected silver catfish juveniles exposed to slightly acidic water (pH 5.0) presented very high lipid peroxidation levels in the liver (988%) at day three compared to uninfected juveniles. This was also observed in infected juveniles maintained at pH 7.0, but to a lesser degree (176%). Consequently, infection by *I. multifiliis* provoked oxidative lipid damage in the liver, and an increase in TBARS levels coincided with progression of the infection. These results are consistent with observations in tambaqui (*Colossoma macropomum* Cuvier), piapara (*Leporinus elongatus* Valenciennes), goldfish (*Carassius auratus* Linnaeus) and silver catfish maintained under hypoxia (Marcon and Wilhelm Filho, 1999; Lushchak et al., 2001; Wilhelm Filho et al., 2005; Braun et al., 2008). As trophonts of *I. multifiliis* infect the gills, respiratory function is likely to be affected, impairing oxygen uptake and causing hypoxia in the tissues. To our knowledge, no studies dealing with this hypothesis were performed. LPO levels in the liver of acará (*Geophagus brasiliensis* Quoy and Gaimard) raised in polluted sites were also increased compared to control fish (Wilhelm Filho et al., 2001).

Infected silver catfish juveniles exposed to pH 7.0 showed lower CAT activity in the liver than uninfected juveniles, probably due to higher increased H_2O_2 degradation in this organ. In polluted sites, the induction of liver CAT activity in acará occurred due to increases in endogenous H_2O_2 levels compared to control fish (Wilhelm Filho et al., 2001). Rays and sharks with high swimming activity also showed higher CAT activity values in the liver compared to rested specimens, corresponding to higher levels of oxygen consumption (Wilhelm Filho and Bovers, 1993). Infected silver catfish juveniles presented higher GST activity at both pH 5.0 and 7.0 compared to uninfected juveniles. GST is involved in the detoxification, and plays an important role in protecting tissue from oxidative stress. A critical role for GST is the defense against oxidative damage and peroxidative products of DNA and lipids (Fournier et al., 1992). Elevated GST activity serves to re-establish the balance between pro-oxidants and antioxidants to alleviate ROS-induced oxidative damage. GST activity in the liver of piapara decreased concurrently with an increase in oxy-

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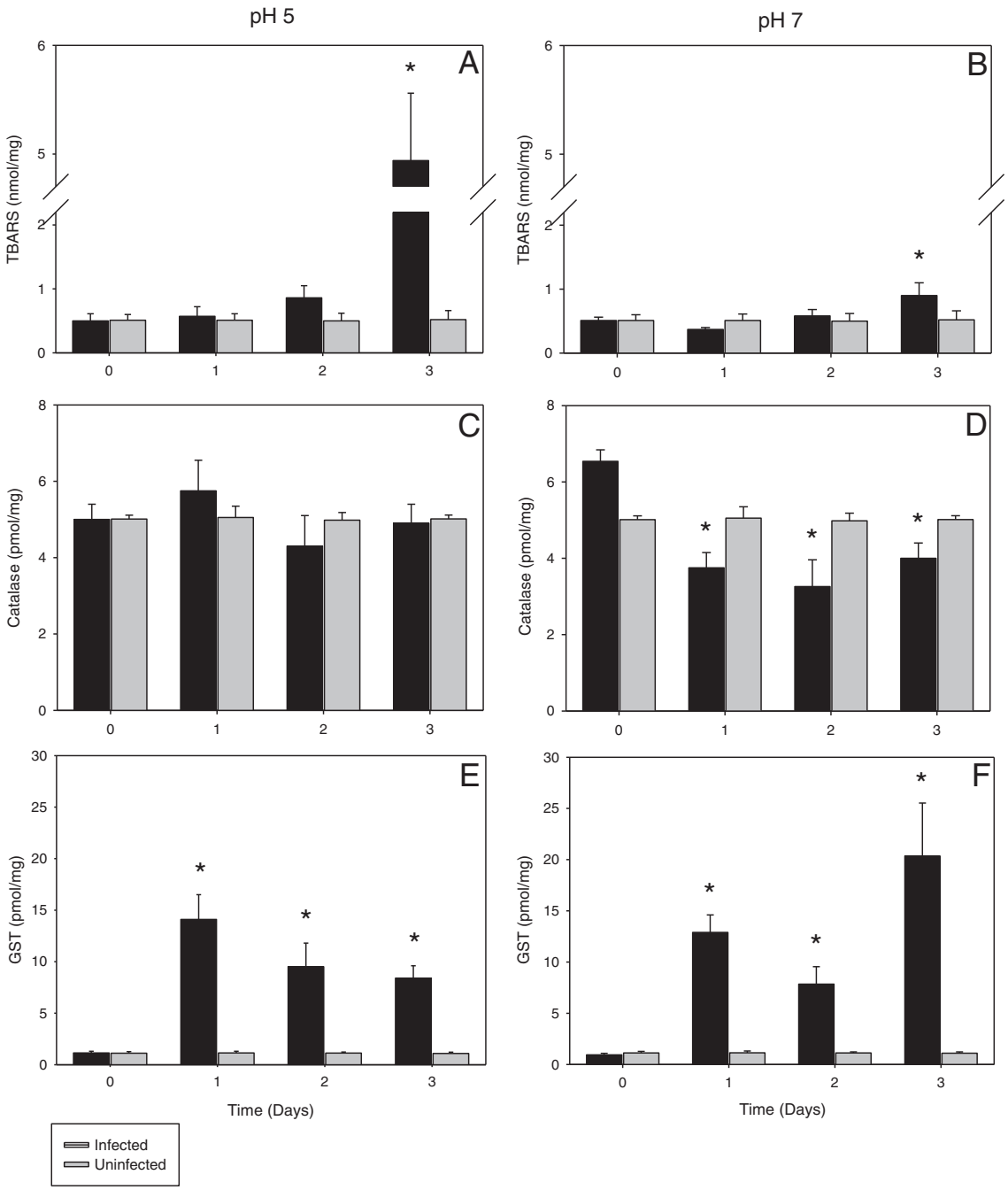

Fig. 1. TBARS, CAT and GST activity in the liver as a function of time (days after the appearance of white spots on the skin of infected juveniles) in uninfected or infected (exposed to *Ichthyophthirius multifiliis*) silver catfish juveniles maintained at pH 5.0 (A, C and E, respectively) and 7.0 (B, D and F, respectively). Values are expressed as means ± SEM, $N = 5$. * Significantly different from uninfected juveniles by two-way ANOVA and Dunnet test ($P < 0.05$).

gen availability, likely due to higher levels of free radicals formed (Wilhelm Filho et al., 2005).

I. multifiliis infection increased lipid peroxidation more than two-fold the in the gills of silver catfish. Lipid peroxidation is a very sensitive marker of oxidative damage in the gills, which suggests that this parasite causes oxidative stress. This effect on lipid peroxidation levels could be explained by direct contact by large numbers of parasites in the gills. The peroxidative damage to gill membranes may result from oxidative deterioration of polyunsaturated fatty acids, thereby impacting the solute and water transport and osmoregulatory functions of gills (Evans, 1987). This process is mediated through free radical metabolites, which affect and alter the cellular antioxidant system. Lipid peroxidation products are not only a marker of oxidative damage, they are also thought to be involved in trigger-

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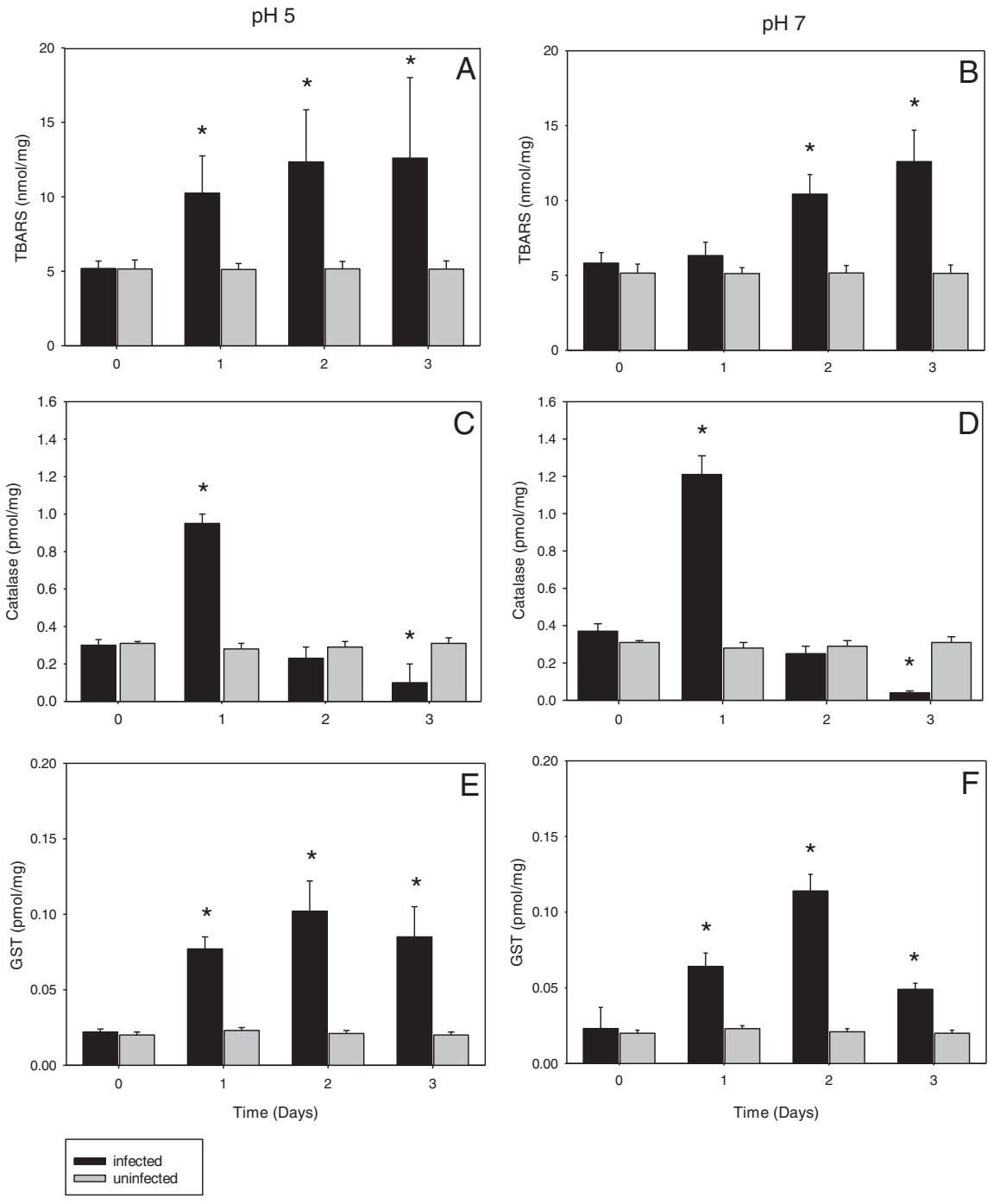

Fig. 2. TBARS, CAT and GST activity in the gills as a function of time (days after the appearance of white spots on the skin of infected juveniles) in uninfected or infected (exposed to *Ichthyophthirius multifiliis*) silver catfish juveniles maintained at pH 5.0 (A, C and E, respectively) and 7.0 (B, D and F, respectively). Values are expressed as means ± SEM, $N = 5$. * Significantly different from uninfected juveniles by two-way ANOVA and Dunnet test ($P < 0.05$).

ing the up-regulation of antioxidant enzymes (Lushchak and Bagnyukova, 2006). Goldfish and piapara exposed to acute temperature changes and moderate hypoxia and normoxia respectively, showed higher lipid peroxide levels, indicative of an increase in pro-oxidant levels (Bagnyukova et al., 2007; Wilhelm Filho et al., 2005). CAT activity in the gills of infected silver catfish juveniles exposed to pH 5.0 and 7.0 increased at day two, but by day three, CAT activity was lower than in uninfected fish. Similar results were found in acará grown in polluted sites compared to controls (Wilhelm Filho et al., 2001). In contrast, no changes in CAT activity were observed in the gills of

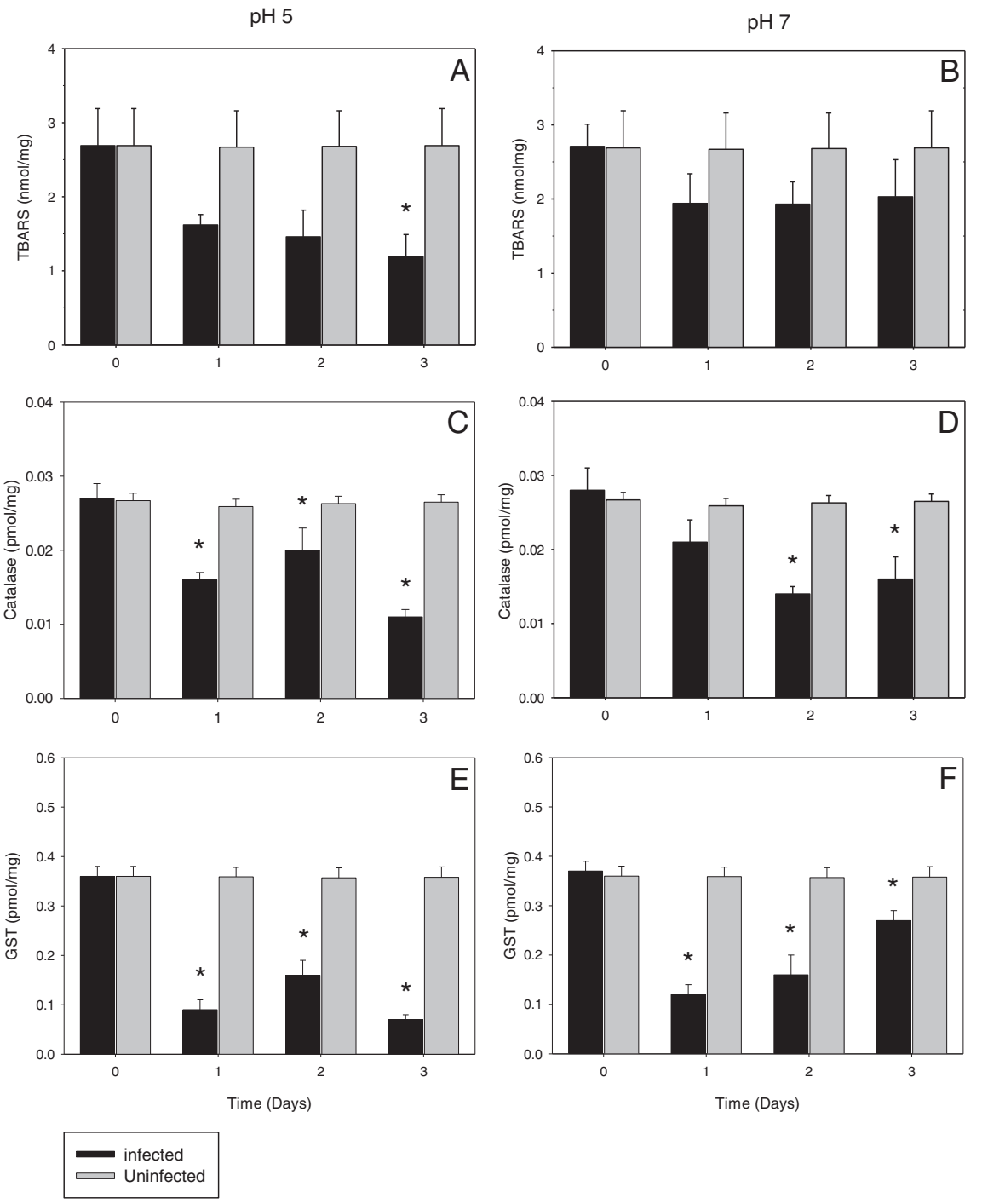

Fig. 3. TBARS, CAT and GST activity in muscle tissues as a function of time (days after the appearance of white spots on the skin of infected juveniles) in uninfected or infected (exposed to *Ichthyophthirius multifiliis*) silver catfish juveniles maintained at pH 5.0 (A, C and E, respectively) and 7.0 (B, D and F, respectively). Values are expressed as means ± SEM, $N = 5$. * Significantly different from uninfected juveniles by two-way ANOVA and test of Dunnet ($P < 0.05$).

Leiostomus xanthurus Lacepède exposed to different dissolved oxygen levels (Cooper et al., 2002). It is likely that the increase in CAT activity in the gills observed in this study indicates an accumulation of H_2O_2 in the gills.

GST activity was higher in the gills of infected silver catfish juveniles compared to uninfected juveniles irrespective of water pH. Increases in the number of parasites is likely related to increases in antioxidant enzyme activity, as oxygen capture is probably reduced due to the presence

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of large numbers of parasites in the gills. Elevated levels of antioxidants enzymes demonstrate that a response occurred in an attempt to neutralize elevated levels of ROS (Monteiro et al., 2009).

Silver catfish juveniles infected with *I. multifiliis* and maintained at pH 5.0 and 7.0 showed lower lipoperoxidation levels, CAT and GST activities in muscle tissues when compared to uninfected juveniles. These results demonstrate that there was no damage to the muscle compared to liver and gills. Silver catfish infected by the parasite *Clinostomum detruncatum* showed a reduction in non-enzymatic antioxidant defenses with respect to pro-oxidant production, leading to oxidative stress and consequent muscle damage via lipid peroxidation products (Belló et al., 2000). Lipid peroxidation levels were higher in muscle tissues of silver catfish, matrinxã (*Brycon cephalus* Günther) and goldfish exposed to hypoxia compared to those maintained in normoxia (Braun et al., 2008; Marcon and Wilhelm Filho, 1999; Lushchak et al., 2001). However, it is possible that lipoperoxidation responses change according to species, given that lipoperoxidation levels in piapara increased in juveniles exposed to moderate hypoxia and normoxia compared to those maintained in severe hypoxia (Wilhelm Filho et al., 2005).

Our results demonstrate that there is an imbalance between pro-oxidant and antioxidant levels in silver catfish juveniles exposed to *I. multifiliis* compared to uninfected fish. This infection induces tissue damage in both the liver and gills, but not in muscle tissues, via lipid peroxidation products. However, elevated antioxidant enzyme activity could indicate a higher degree of liver and gill protection against *I. multifiliis*.

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