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Benefits of using the probiotic Efinol® L during transportation of cardinal tetra, *Paracheirodon axelrodi* (Schultz), in the Amazon

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Abstract

The objective of this experiment was to test the probiotic Efinol® L during transportation of cardinal tetra, *Paracheirodon axelrodi* (Schultz). For the transportation, fish were distributed in 18 plastic tanks, of which nine contained the Efinol® L (10 mg L^{-1}; probiotic treatment) and the remaining had no probiotic (control treatment). Transport lasted 24 h and three different boxes of each treatment were sampled at 3, 12 and 24 h. Up to the 12-h sampling period, no significant difference in the survival was observed; on the other hand, survival was higher at the end of the transport (24 h) in the probiotic treatment. No significant difference was found in dissolved oxygen and temperature between treatments. Conductivity, pH and alkalinity increased along the transport, but without a difference between treatments. Ammonia increased in all treatments, although it was significantly lower in the probiotic group at 24 h. Cortisol levels were significantly higher in all transported fish when compared with the basal values. At 24 h, cortisol levels in control fish were significantly higher than those in the probiotic treatment. With the observed results, we are able to conclude that the probiotic Efinol® L is efficient during cardinal transport, lowering the mortality and helping maintain water quality possibly by lowering metabolic wastes.

Keywords: probiotic, survival, Amazon, ornamental fish, ion flux, body cortisol

Introduction

The cardinal tetra, *Paracheirodon axelrodi* (Schultz), is an endemic fish species from the middle Rio Negro Basin widely used as an ornamental fish in aquariums worldwide (Walker 2004). The cardinal tetra is the most important commercial species of ornamental fish exported from the Brazilian Amazon, contributing to about 80% of exports. Some 12–15 million wild specimens are legally exported annually and accounted by the Brazilian government (Chao 2001). However, Chao and Prada-Pedreros (1995) estimate that at least 24–32 million cardinals are taken from the Rio Negro tributaries each year. The ornamental industry generates 60% of the total income of the middle Rio Negro region's economy (Chao & Prang 1997), with between 6000 and 8000 fishermen involved in this activity (Chao & Prada-Pedreros 1995; Prang 2001).

Transportation is an important part of all phases of the export process. Cardinals are normally caught in small streams (igarapé). After this, fish are typically transported in 40-L^{-1} plastic boxes to Barcelos, a base city for the ornamental industry in the middle

Rio Negro region, then onto exporters' facilities in Manaus and finally to importing countries, mainly Europe, Asia and North America (Santos & Santos 2005). According to Waichman, Silva and Marcon (2001), mortality during the transportation between Barcelos and Manaus is a negative factor in the exporting process, which can be attributed to the problem of bad management practices and poor water quality leading to disease outbreaks, mostly bacteria based.

Most recently, probiotics have appeared in the market as a method to increase the quality and health of several fish species during some processes involved in management (Gatesoupe 1999; Gomez-Gil, Roque & Turnbull 2000). These products are primarily composed of highly concentrated bacteria, vitamins and nutrients (Gatesoupe 1999; Verschuere, Rombaut, Sorgeloos & Verstraete 2000) and were created to stimulate the immune system and help increase the water quality in the environment. The use of probiotic helps increase fish health during stressful processes such as severe changes in environmental conditions (Taoka, Maeda, Jo, Jeon, Bai, Lee, Yuge & Koshio 2006). Carnevali, De Vivo, Sulpizio, Gioacchini, Olivotto, Silvi and Cresci (2006) observed an inverse correlation between gut colonization with lactic acid bacteria strain and cortisol levels; thus, Rollo, Sulpizio, Nardi, Silvi, Orpianesi, Caggiano, Cresci and Carnevali (2006) suggest that a probiotic is able to modulate cortisol responses in fish, lowering the magnitude of stress response.

Most probiotics are designed to be mixed with food, but recently some probiotic products have been formulated that can be dissolved in water and can help during management and transport operations. Efinol® L is a water-soluble commercial product sold as a proven anti-stress formula for aquaculture hatcheries, useful for larval transport and to improve the health and survival of aquatic animal larvae. This product is formulated with *Bacillus subtilis*, *Bacillus licheniformes*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae*, along with selected amino acids, vitamins, minerals, free-flow and anti-caking agents (calcium carbonate and silica). This combination characterizes the product as a probiotic enriched with some nutrients. Previous results obtained by Gomes, Brinn, Marcon, Dantas, Brandão, Abreu, McComb and Baldisserotto (2008) during transportation of another Amazonian ornamental fish, the marbled hatchetfish, *Carnegiella strigata* (Günther), with Efinol® L demonstrated that this probiotic was efficient in improving the water quality by the reduction in metabolic wastes and reduction in stress responses. Therefore, the objective of this experiment was to test the efficiency of Efinol® L solution during the transport of the cardinal tetra between Barcelos and Manaus.

Material and methods

This work was carried out in December 2005 during a scientific expedition to the middle Rio Negro region, close to Barcelos, AM, Brazil, aboard the Miss Bebel vessel, with the main objective of studying the biology, fishing and management of several ornamental fish captured and exported from this region.

Cardinals (0.032 ± 0.011 g; mean ± SD) were captured by professional fishermen in a small stream called igarapé Puxurituba. After capture, the fishermen used a standard procedure for keeping the fish before transport to Barcelos. Fish were kept in plastic boxes containing 20 L of water before the actual transport. Between the capture and the experimental period, fish were maintained for a maximum of 10 days in boxes and fed with a commercial feed for omnivorous fish (32% crude protein and 4180 kcal CE kg^{-1} of crude energy) twice a day. Fish were starved 2 days before the transport.

For the transport experiment, 18 plastic boxes were utilized with a total capacity of 40 L but filled with 10 L of local stream water. Fish were randomly distributed between the boxes following the procedure used by local fishermen, with a final density of 578 ± 26 cardinals per box or 1.84 ± 0.19 g of fish per litre of water. The density used was in the range (500–1000 per box) commercially used by local fishermen. In half of the boxes, a solution of 10 mg L^{-1} probiotic Efinol® L (Bentoli Agrinutrition, Austin, TX, USA) was utilized, while the rest were maintained with probiotic-free water (control group). The probiotic concentration is in accordance with the manufacturer's instructions. A 24-h transport experiment proceeded with the vessel simulating conditions that local fishermen use to take their fish through Barcelos to Manaus. Survival, water quality, net ion flux and body cortisol were monitored from three different boxes of each treatment at 3, 12 and 24 h of transport. Each box was sampled only once and then discarded from the experiment.

For whole-body cortisol analysis, samples of unstressed fish were caught directly from the local streams to be compared with the transported fish. They were caught and anaesthetized with a lethal

concentration of benzocaine (200 mg L^{-1}) and immediately frozen and stored in liquid nitrogen. During transport, fish samples were taken from each box at each sampling time and frozen in liquid nitrogen.

Water analysis of temperature, dissolved oxygen (YSI 55), pH (YSI pH-100) and conductivity with a digital meter (Bernauer, Blumenau, Brazil) was performed directly in the box. Water samples were taken from the box for further analysis of alkalinity by titration (American Public Health Association, American Water Works Association, Water Environment Federation 1992), total ammonia by the salicylate method (Verdouw, Vanechted & Dekkers 1978) and net ion fluxes of Na^+, K^+ and Cl^-.

Water Na^+ and K^+ levels were measured directly in a B462 flame photometer (Micronal, Brazil) and Cl^- levels using the colorimetric assay described by Zall, Fisher and Garner (1956). Net ion fluxes (J_{net}) were calculated based on changes in the ion concentration of transport water over the sampling periods according to the equation by Gonzalez, Wood, Wilson, Patrick, Bergman, Narahara and Val (1998): $J_{net} = V([ion]_1 - [ion]_2) \times (Mt)^{-1}$, where $[ion]_1$ and $[ion]_2$ are the bath ion concentrations at the beginning and the end of the flux period, respectively, V is the bath volume in litres, M is the mass of the fish in kg and t is the duration of the flux period in hours. Initial waterborne Na^+ levels in the fish boxes were (μmol L^{-1}, mean ± SEM) 16.5 ± 0.4 and 43.4 ± 2.1, Cl^- 32.0 ± 6.0 and 84.6 ± 12.4 and K^+ 2.6 ± 0.3 and 18.2 ± 1.7 for control and probiotic groups respectively.

Whole-body cortisol extractions were performed according to de Jesus, Hirano and Inui (1991) with a few modifications. Briefly, pools of 10 fish were homogenized with phosphate-buffered saline with gelatin (PBS-G) and a 0.5 g sample was taken for extraction. The samples underwent a triple extraction using diethyl ether, followed by evaporation in a water bath and further resuspension of the pellet with PBS-G before assay. Radioimmunoassay was performed using a commercial kit (DPC®) for cortisol in humans. A thorough validation through parallel curves and extraction efficiency process was performed for this species. The limit of detection was 0.2 μg dL^{-1} and intra-assay and inter-assay coefficients of variation were below 8%. The average extraction efficiencies varied from 49% to 57%, and the values reported in this article were not corrected for efficiency.

Homogeneity of variances among the different groups was tested using the Levene test. Data of treatment groups presented homogeneous variances and survival was compared using a *t*-test, while water quality and net ion fluxes were compared using a two-way ANOVA (time and treatment as variables) and the Tukey test. Differences between treatments and body cortisol at different samplings were compared with unstressed fish (basal values) using one-way ANOVA and Dunnett's test ($P < 0.05$) and between treatments at the same sample time using a *t*-test. All statistical tests were conducted using software STATISTICA (version 5.1). Data were expressed as means ± SEM, and the minimum significance level was set at $P < 0.05$.

Results

After 12 h of transport, less than 1% of fish died in both the treatments; on the other hand, significant mortality between treatments occurred in the 24-h sampling period, with the probiotic treatment having the higher survival (99.71 ± 0.29%) when compared with control (94.62 ± 1.44%) (Table 1).

No significant differences were observed in dissolved oxygen levels and temperature (Fig. 1) between treatments, although levels of oxygen decreased to levels close to or below 2 mg L^{-1} after 3 h of transport, maintaining these levels throughout the experiment. Temperature varied between 28.5 and 30.2 °C. Conductivity, pH and alkalinity increased significantly during the transport process (Fig. 1). At the end of the transport, conductivity was 37.87 ± 0.76 and 38.43 ± 4.14 μs cm^{-1} in the probiotic and control treatment respectively (Fig. 1). The pH did not change significantly between treatments; however, it increased significantly along the transport periods. The initial pH was 4.9–5.0 and reached 6.50–6.58 at the end (Fig. 1). Alkalinity was significantly higher in the probiotic treatment after 24 h

Table 1 Survival of cardinal tetra (*Paracheirodon axelrodi*) during transportation with (probiotic Efinol® L treatment) and without addition of probiotic to the water (control treatment)

Time of transport (h)	Control (%)	Probiotic (%)
3	99.82 ± 0.18	99.77 ± 0.14
12	99.40 ± 0.35	99.94 ± 0.06
24	94.62 ± 0.83	99.71 ± 0.16*

$N = 3$ boxes per treatment at each sample time.
*Indicates significant difference from control by *t*-test ($P < 0.05$).

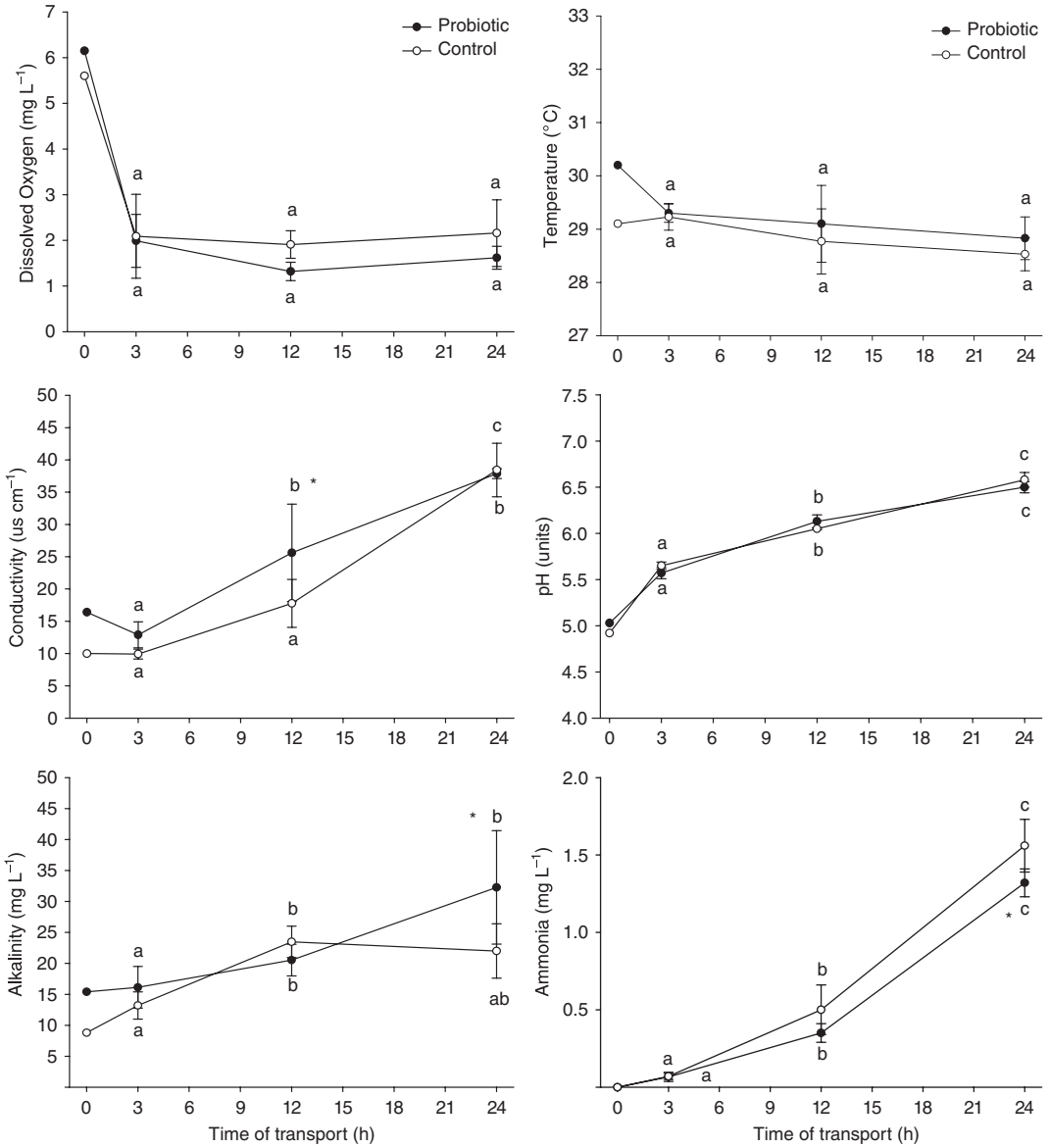

Figure 1 Water parameters during transportation of cardinal tetra (*Paracheirodon axelrodi*) with (Probiotic Efinol® L treatment) and without addition of a probiotic to the water (control treatment). $N = 3$ boxes per treatment at each sample time. Asterisk (*) indicates significant differences from control by two-way ANOVA and the Tukey test ($P < 0.05$). Different letters indicate significant differences among times of transport within the same group by two-way ANOVA and the Tukey test ($P < 0.05$).

(32.27 ± 9.16 mg L^{-1}) while it reached 22.00 ± 4.40 mg L^{-1} in the control group (Fig. 1). Total ammonia increased with transport time, reaching significantly higher levels in the control group at the 24-h sampling. At 24 h, ammonia concentrations were 1.56 ± 0.17 mg L^{-1} in the control group and 1.32 ± 0.09 mg L^{-1} in the probiotic group (Fig. 1).

Control cardinal tetras presented a net Na^+ influx and net Cl^- and K^+ effluxes after 3 h of transport. After 12 h of transport, net Na^+ influx reverted to a net efflux, while net Cl^- efflux reverted to a net influx (but much lower than the previous net efflux). These fluxes remained constant up to 24 h (Fig. 2a and b). Net K^+ flux did not change throughout the

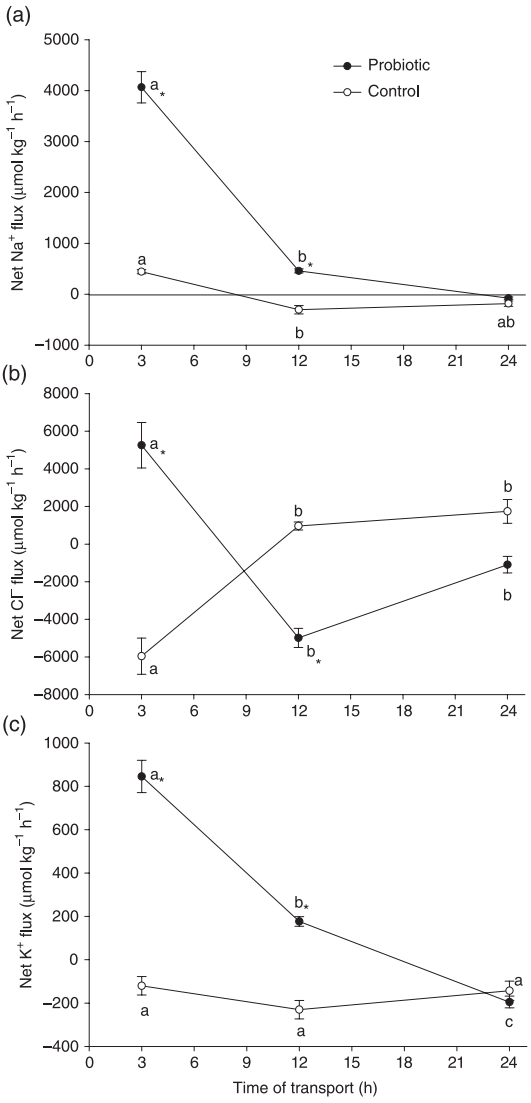

Figure 2 Net Na^+ (a), Cl^- (b) and K^+ (c) fluxes of cardinal tetra (*Paracheirodon axelrodi*) during transportation with (Probiotic Efinol® L treatment) and without addition of a probiotic to the water (control treatment). Positive values indicate net influxes and negative values indicate net effluxes. $N = 3$ boxes per treatment at each sample time. Asterisk (*) indicates significant differences from control by two-way ANOVA and the Tukey test ($P < 0.05$). Different letters indicate significant differences among times of transport within the same group by two-way ANOVA and the Tukey test ($P < 0.05$).

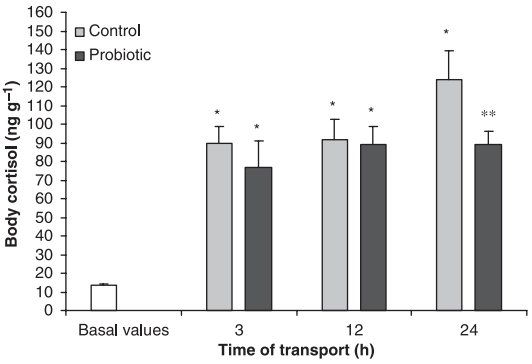

Figure 3 Body cortisol concentrations of cardinal tetra (*Paracheirodon axelrodi*) during transportation with (Probiotic Efinol® L treatment) and without addition of a probiotic to the water (control treatment). $N = 3$ boxes per treatment at each sample time. Asterisk (*) indicates a significant difference from the basal values by one-way ANOVA and Dunnett's test ($P < 0.05$). Double asterisk (**) indicates significant differences between treatments at each sampling time using a *t*-test ($P = 0.049$).

transport (Fig. 2c). Cardinal tetras exposed to a probiotic showed a very high net influx of all measured ions after 3 h of transport. These net ion influxes reduced significantly after 12 h of transport, but net Na^+ and K^+ influxes were still significantly higher than those of control fish, while Cl^- presented a very high net efflux. All ion fluxes tended to zero and were not significantly different from ion fluxes in control cardinal tetras after 24 h (Fig. 2).

Whole-body cortisol levels were significantly lower in the baseline group when compared with all samples from the transport experiment. The baseline value was 13.75 ± 0.47 ng g^{-1} (Fig. 3). All transported fish samples had cortisol levels at least fourfold higher than the baseline levels, some reaching an eight- to nine-fold difference. Body cortisol was significantly greater in control fish than in fish from the probiotic treatment at 24 h of transport (Fig. 3).

Discussion

Wild-caught fish represent only 10% of the freshwater ornamental fish commerce in the world (Tlusty 2002), and the Amazon basin is one of the main sources (Gerstner, Ortega, Sanchez & Graham 2006). Cardinal tetra is one of the most exploited freshwater ornamental fish species in the world and the wild stocks are declining (Bayley & Petrere Jr 1989). The use of Efinol® L improved fish survival during transportation, which may represent an additional survival of 1.2–1.6 million fish per year. This result is important from a commercial and environmental point of view, because it could potentially improve the gain of fishermen and reduce fish exploitation,

which will help to maintain the wild stock. One additional advantage of the use of Efinol® L is the low cost; a dosage used in one plastic box filled with 10 L of water (100 mg) costs less than two cents in US dollars.

The explanation for the negligible mortality of probiotic-treated fish at 24 h is probably related to cortisol. Basal cortisol levels are at least eightfold smaller than any other value during the entire experimental period, demonstrating a high stress response in all treatments and times of transport, with greater values in control fish at 24 h. High levels of cortisol lead to immunosuppression and consequent exposure to pathogens could lead to mortality [reviewed by Rollo *et al.* (2006)]. Efinol® L might have worked in two ways: by diminishing the stress stimulus or affecting the physiology of stress response by modifying clearance in cortisol in the body or by decreased production from the interrenal gland.

The two principal mechanisms of action of the probiotics described in fish are (1) as an antagonist to pathogens (Verschuere *et al.* 2000) and (2) through stimulation of the immune system (Taoka *et al.* 2006). Water-diluted probiotics have been demonstrated to improve the survival of halibut *Hippoglossus hippoglossus* (Linnaeus), turbot, *Scophthalmus maximus* Linnaeus, and Atlantic salmon, *Salmo salar* Linnaeus, when exposed to pathogenic bacteria and channel catfish, *Ictalurus punctatus* (Rafinesque), during the grow-out period in ponds [reviewed by Verschuere *et al.* (2000)].

According to Gatesoupe (1999), several commercial products have sought to exploit the same idea that bacteria, which improve water quality, may be beneficial to animal health. These products are referred to as 'probiotics' and most of them contain nitrifying bacteria and/or *Bacillus* spp. These two kinds of bacteria are quite different. The nitrifying bacteria have strict ecological niches, their actions interfere directly with the water quality and they have not been detected in the gastrointestinal tract of animals. The strains of *Bacillus* spp. used as probiotics may be active in the intestine during transit, with an indirect action on the water quality (Gournier-Chateau, Larpent, Castellanos & Larpent 1994).

The water used for fish transportation was characterized as black water, and some studies demonstrate that the black waters from Rio Negro Basin are among those with the lowest mineral levels in the world (Mortatti & Probst 2003). In several characid species that live in this environment, ion regulation is characterized by high-affinity and high-capacity ion transport systems (Gonzalez, Dalton & Patrick 1997; Gonzalez & Preest 1999). The same was observed in the Na^+ transport system of cardinal tetras, which is specialized for high rates of Na^+ uptake even in the dilute waters of Rio Negro (Gonzalez & Wilson 2001). At the beginning of the transport, cardinal tetra presented a net influx (gain) of Na^+ and a very high net efflux (loss) of Cl^-. Both fluxes tended to near-zero values after 24 h, i.e., equilibrium between influx and efflux. Cardinal tetras exposed to probiotic showed a very high net influx of ions at the beginning of the transport. This was expected because the addition of probiotic to the water increased Na^+ and Cl^- levels by 2.6-fold and K^+ levels by ninefold, and according to Gonzalez, Wilson, Wood, Patrick and Val (2002), at least for Na^+, the increase in waterborne Na^+ levels led to a higher increase in the Na^+ influx compared with efflux in cardinal tetras, which would result in a higher net Na^+ influx. In spite of this, net ion fluxes in cardinal tetras maintained in water with a probiotic also tended to equilibrium after 24 h, demonstrating a fast adjustment to the higher waterborne ion levels.

Before transport, dissolved oxygen levels were 5.7–6.0 mg L^{-1}, values similar to those measured in the igarapé where fish were collected during our expedition. However, Geisler and Annibal (1984) reported oxygen concentrations between 1 and 5.7 mg L^{-1} in some igarapés where cardinal tetras occur. After 3 h of transportation, oxygen levels declined to 2 mg L^{-1} and these levels were maintained throughout till the end of transportation. These oxygen concentrations can cause mortalities in some fish species during transportation (Berka 1986), but not for cardinals, as these values are similar to those observed in the wild. To support this, the low oxygen concentrations were similar in both treatments, and fish from probiotic treatment presented negligible mortality. The maintenance of dissolved oxygen levels at a constant level through 3–24 h of transport could be explained by the huge water surface in the fish boxes, which allows enough gaseous exchange to maintain these oxygen levels.

The temperatures measured during transportation are in accordance with the ones reported by Froese (1998) during transportation of tropical fish, and probably did not cause any adverse effect. The increase in pH, alkalinity and conductivity during transportation is mainly associated with ammonia excretion and ion fluxes, which contribute to the growth of salt availability in the water. The increase in pH levels in the present work is in contrast with those reported when fish are transported in closed systems (plastic bags into

styrofoam boxes), in which a progressive decrease in pH occurs due to CO_2 accumulation in the water (Lim, Dhert & Sorgeloos 2003). Because the plastic boxes that transport cardinal tetras are in direct contact with air, the CO_2 produced and excreted by fish should not contribute to the observed changes in water pH during transportation. In addition, while in closed systems, declines in pH and accumulated CO_2 and ammonia may be critical for fish health and survival, in the transport procedure used for Amazonian fish, including cardinal tetra, wasted ammonia accumulation might deserve more attention during water quality monitoring, whether due to its own toxic effect to the fish or due to the resulting changes in water pH.

The probiotic treatment presented a lower ammonia concentration than the control treatment. Gomes *et al.* (2008) also observed a lower ammonia concentration in the water of marbled hatchetfish transported with Efinol® L. During stressful situations, the demand for energy increases and cortisol helps maintain energy levels by utilizing sources such as glycogen and eventually breakdown of protein that would generate ammonia. Therefore, the main reason for the lower ammonia excretion in the probiotic treatment at 24 h is the reduction in metabolic wastes. On the other hand, the higher ammonia concentration in the control group at 24 h is mainly induced by the increase in cortisol. The concentrations of ammonia in both treatments at the end of transportation are lower than the pattern observed in the majority of fish transportation procedures described (Berka 1986; Lim *et al.* 2003), and these values are associated with the lower fish density in the fish boxes and an adequate fasting period before transportation.

Whole-body cortisol levels of unstressed cardinal tetras were higher than those found in zebrafish, *Danio rerio* (Hamilton) (Ramsay, Feist, Varga, Westfield, Kent & Schreck 2006), and in three-spined stickleback, *Gasterosteus aculeatus aculeatus* Linnaeus (Pottinger, Carrick & Yeomans 2002), but similar to those in other Amazonian ornamental fish, the marbled hatchetfish (Gomes *et al.* 2008). The significantly higher cortisol levels found in transported fish compared with levels of fish caught in the environment demonstrate that Efinol® L is not efficient in suppressing stress responses in fish in a 24-h period, which is in agreement with the ion flux results. However, fish from the probiotic treatment presented a lower magnitude of stress than control fish at 24-h sampling. This lower cortisol response in probiotic-treated fish was also observed by Gomes *et al.* (2008) during transportation of marbled hatchetfish and by Rollo *et al.* (2006) during pH-induced stress in seam bream, *Sparus auratus* Linnaeus. Cortisol levels in fish transported with probiotic were stable throughout the transport procedure, whereas in control fish, cortisol concentration increased at 24 h, which was in agreement with the hypothesis that high cortisol levels were the main reason for the fish mortality in the control treatment.

The results show that Efinol® L is efficient in reducing fish mortality, stress response and improving the water quality during transportation and that it should be used during transportation of fish species. However, it is also recommended that the amounts of ions in the probiotic be reduced for use with fish from ion-poor waters, in an effort to decrease osmoregulatory disturbances.

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