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Using Efinol® L during transportation of marbled hatchetfish, *Carnegiella strigata* (Günther)

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Abstract

The objective of this experiment was to test the efficacy of a probiotic (Efinol® L) during transportation of marbled hatchetfish, *Carnegiella strigata*. Wild specimens were captured from a small stream and transported for 24 h in plastic fish boxes with a probiotic (10 mg L^{-1}) and probiotic-free water. The boxes were sampled at 3, 12 and 24 h of transport. At the end of the experiment, the survival rate was close to 100% in both treatments. Dissolved oxygen diminished with time in both treatments, but the probiotic group had significantly higher levels. Conductivity, pH and ammonia increased significantly during the transport, demonstrating higher levels in the probiotic-free group. Fish from both treatments presented very high net Na^+ and K^+ effluxes after 3 h of transport. At 24 h, net K^+ effluxes in fish of the probiotic treatment reached values close to zero and a significantly lower Na^+ efflux was observed. Cortisol levels in both treatments at 3 and 12 h were significantly higher than that in control samples. Higher body cortisol levels were observed in the probiotic-free group than that in the probiotic group at 3 and 12 h. The results demonstrate that addition of a probiotic during fish transport improves water quality and leads to fish presenting a lower stress response intensity.

Keywords: probiotic, fish health, water quality, stress, ion flux, body cortisol

Introduction

Ornamental fishery is the most important economic activity of the middle Rio Negro, Amazon, Brazil, and generates 60% of the total income of the region (Chao & Prang 1997). According to Prang (2001), there are between 6000 and 8000 fishermen involved in this activity. Around 180 freshwater fish species are legally exported from the Brazilian Amazon as ornamentals each year. The main exported fish are Cardinal tetra, *Paracheirodon axelroldi* (Schultz), Neon tetra, *Paracheirodon innesi* (Myers), Bleeding heart tetra, *Hyphessobrycon erythrostigma* (Fowler), Firehead tetra, *Hemigrammus blehery* G.&M., marbled hatchetfish, Adolf's catfish, *Corydoras adolfoi* B., and sting rays species of the family Potamotrygonidae (Freitas & Rivas 2006).

Fish are normally caught from small streams (igarapés), flooded forest (igapó) or lakes (Chao & Prada-Pedreros 1995) and transported to an improvised fishing camp, where they are maintained in small cages. After this, fish are typically transported in 40 L plastic boxes containing water to only one-fourth of their capacity, to Barcelos, a base city for the ornamental industry in the middle Rio Negro region, then to exporter's facilities in Manaus (Santos & Santos 2005) and from there to import countries. The transportation by boat between Barcelos and Manaus takes approximately 24 h, which encompasses 500 km of river, and is the most critical phase of the ornamental fish export process within the Amazon region. The successful transport of fish in this phase is difficult for several reasons including (1) fish are normally stressed and under-fed at the inception of transport and (2) poor water quality and management procedures during this transport procedure (Waichman, Silva & Marcon 2001). Consequently, high mortality rates during this phase of the export process are common (Leite & Zuanon 1991).

During transportation between Barcelos and Manaus, fishermen's standard procedure is to renew 50–100% of the water in the boxes in an effort to improve the water quality. However, Waichman *et al.* (2001) reported that this procedure has little effect on the water quality and that manipulation of the boxes can cause additional stress in fish. Table salt is also utilized to mitigate stress during transportation of some species, such as cardinal, marbled hatchetfish and discus *Symphysodon discus* H.; however, there is no standardized concentration and excess use may cause osmoregulatory disturbance (Waichman *et al.* 2001).

Probiotics that increase the quality and health of fish species have recently appeared in the market (Gatesoupe 1999; Gomez-Gil, Roque & Turnbull 2000). Some probiotic products have been formulated to dissolve in water during transport operations. Efinol®L is a water-soluble commercial product sold as a proven anti-stress formula for aquaculture hatcheries, which is useful for larval transport, and to improve the health and survival rate 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. This combination characterizes the product as a probiotic enriched with nutrients.

Marbled hatchetfish represents one of the largest exports of freshwater ornamental fish from the middle Rio Negro region (Freitas & Rivas 2006) and comprises 2% of the total import of freshwater ornamental fish in the United States (Chapman, Fitz-cox, Thunberg & Adams 1997). Therefore, the objective of this experiment was to test the efficiency of Efinol®L solution during the transport process of marbled hatchetfish between Barcelos and Manaus.

Materials and methods

This work was developed during a scientific expedition to the middle Rio Negro region, close to Barcelos, Amazon, Brazil, aboard the Miss Bebel vessel, with its main objective being to study the biology and management of several ornamental fish captured and exported from this region.

Marbled hatchetfish (0.39 ± 0.01 g, mean ± SE) were captured by professional fishermen from a small stream called igarapé Puxurituba. After capture, the fishermen used their standard procedure for storing fish before transport to Barcelos. Fish were kept in a 2 m^3 cage inside the igarapé before the actual transport. Between capture and the experimental period, fish were maintained for a maximum of 10 days in a cage.

For the transport experiment, 18 plastic boxes were utilized, with a total capacity of 40 L filled with 10 L of local stream water. Fish were randomly distributed in the boxes following the procedure used by local fishermen, with a final density of 214 ± 9.18 hatchetfish per box or 8.36 ± 0.36 g of fish L^{-1} of water. This is the density used locally in the commercial transport procedure (Waichman *et al.* 2001). In half of the boxes, a solution of 10 mg L^{-1} probiotic Efinol®L (Bentoli Agrinutrition, Austin, TX, USA) was utilized, while the remaining boxes were maintained with probiotic-free water (control group). The probiotic concentration was in accordance with the manufacturer's instructions. A 24-h transport experiment was carried out between Barcelos and Manaus under the same conditions in which fish are normally commercially transported. Survival, water quality, net ion fluxes 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 environment (local streams) with minimum disturbance to in compare them with the transported fish. They were caught and anaesthetized with a lethal dose of benzocaine and immediately frozen and stored in liquid nitrogen. During transport, fish samples (triplicates of 10 fish) were taken from each box at each sampling time and frozen in liquid nitrogen.

Water analysis for temperature, dissolved oxygen (YSI 55, YSI, Yellow Springs, OH, USA), pH (YSI pH-100, YSI) and conductivity using a digital meter was carried out directly in the box. Water samples were taken from the box for further analysis of alkalinity using titration (American Public Health Association, American Water Works Association, Water Environment Federation 1992), total ammonia using the salicylate method (Verdouw, Vanechted & Dekkers 1978) and net ion fluxes of Na^+ and K^+. Water Na^+ and K^+ levels were measured directly in a B462 flame photometer (Micronal, São Paulo, Brazil). Net ion fluxes were calculated according to Gonzalez, Wood, Wilson, Patrick, Bergman, Narahara and Val (1998). The initial waterborne Na^+ levels in the fish boxes were 2.7 ± 0.2 and 12.0 ± 0.3, K^+ 2.2 ± 0.2 and 10.3 ± 0.2 (μmol L^{-1}, mean ± SEM) 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 and a 0.5 g sample was taken for extraction. A triple extraction using diethyl ether and further radioimmunoassay were performed using a commercial kit (DPC®, Diagnostic Products, Los Angeles, CA, USA) for cortisol in humans. The assay was validated for this species by performing a parallelism test and calculating the extraction efficiency. The mean extraction efficiencies varied from 51% to 57%, and the values reported are not corrected for efficiency.

Homogeneity of variances among the different groups was tested using the Levene test. Data from Na^+ fluxes did not present homocedastic variances and were subjected to inverse arcsine transformation before statistical analysis. Water quality and net ion flux values were compared using a two-way ANOVA (time and treatment as variables) and multiple comparisons using Tukey *post hoc* tests. Differences between treatments and body cortisol were compared with unstressed fish (basal values) using Kruskal–Wallis ANOVA on ranks and Dunn's test ($P < 0.05$), and between treatments at each sampling time using a *t*-test. All statistical tests were conducted using software STATISTICA (version 5.1), and data are expressed as mean ± SD with the minimum significance level set at $P < 0.05$.

Results

No mortality occurred in the 3- and 12-h samples in any of the treatments, and at the end of the experiment, the survival rates were 99.77 ± 0.23% and 99.73 ± 0.24% for control and probiotic treatments respectively. Dissolved oxygen decreased with time in both treatments, while the probiotic treatment had significantly higher levels (Table 1). After 3 h of transportation, oxygen levels were 4.04 ± 0.26 and 3.62 ± 0.31 mg L^{-1}, with the higher levels being in the probiotic group. At the end of the transport (24 h), oxygen concentrations reached 3.06 ± 0.31 mg L^{-1} in the probiotic group and 2.69 ± 0.25 mg L^{-1} in the control group. Temperature varied significantly during the experiment, reaching its highest levels in the 24-h sampling period (Table 1). The temperature was recorded at 28.4 °C at the beginning of the transport and reached 29.23 ± 0.80 and 29.70 ± 0.62 °C at 24 h in the probiotic and control treatments respectively.

Conductivity, pH and ammonia increased significantly along the transport, demonstrating higher levels in the probiotic-free group (Table 1). Conductivity was found to be 10.05 μS cm^{-1} at the beginning of the experiment, 10.9 ± 0.62 and 13.83 ± 0.57 μS cm^{-1} at the 3-h sampling period and 25.2 ± 4.44 and 31.33 ± 3.26 μS cm^{-1} at the end of the transport in the probiotic and control treatment respectively. The initial pH was 4.91 and increased

Table 1 Two-way ANOVA of water quality during transportation of marbled hatchetfish (*Carnagiella strigata*) with (probiotic Efinol®L treatment) and without (control treatment) addition of Efinol®L

Source	d.f.	Sum of squares	Mean square	*F*-ratio	*P*
Dissolved oxygen (mg L^{-1})					
Time	2	7.733	3.867	15.905	0.000
Treatment	1	1.417	1.417	5.828	0.033
Interaction	2	0.255	0.127	0.524	0.605
Error	12	2.917	0.243		
Temperature (°C)					
Time	2	42.510	21.255	72.874	0.000
Treatment	1	0.201	0.201	0.688	0.423
Interaction	2	0.214	0.107	0.368	0.700
Error	12	3.500	0.292		
Conductivity (μS cm^{-1})					
Time	2	793.240	396.620	49.382	0.000
Treatment	1	61.976	61.976	7.716	0.017
Interaction	2	13.764	6.882	0.857	0.449
Error	12	96.380	8.032		
pH (units)					
Time	2	1.411	0.705	113.462	0.000
Treatment	1	0.128	0.128	20.647	0.001
Interaction	2	0.036	0.018	2.920	0.093
Error	12	0.075	0.006		
Alkalinity (mg L^{-1})					
Time	2	66.684	33.342	12.400	0.001
Treatment	1	1.076	1.076	0.400	0.539
Interaction	2	2.151	1.076	0.400	0.679
Error	12	32.267	2.689		
Total ammonia (mg L^{-1})					
Time	2	2.718	1.359	30.673	0.000
Treatment	1	0.368	0.368	8.305	0.016
Interaction	2	0.011	0.006	0.125	0.884
Error	12	0.443	0.044		

Interaction = time × treatment.

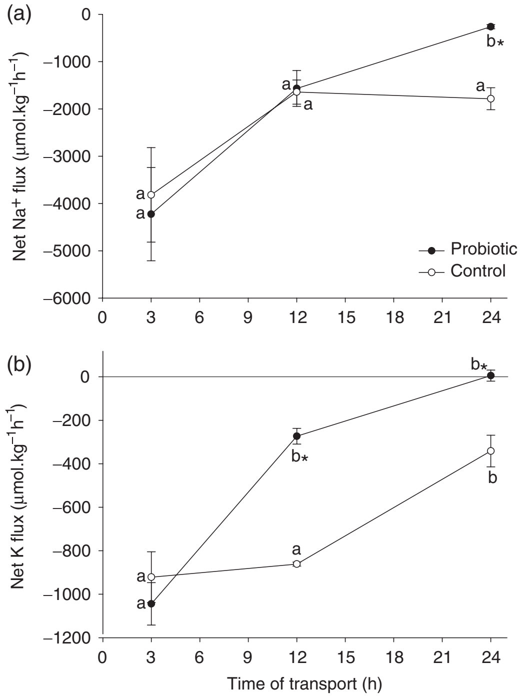

Figure 1 Net Na$^+$ (a) and K$^+$ (b) fluxes of marbled hatchetfish (*Carnegiella strigata*) during transportation with (probiotic treatment) and without addition of Efinol®L in the water (control treatment). Positive values indicate net influxes and negative values indicate net effluxes. *Significant difference from the control using two-way ANOVA and Tukey's test ($P < 0.05$). Different letters indicate significant difference among times of transport within the same group using two-way ANOVA and Tukey's test ($P < 0.05$).

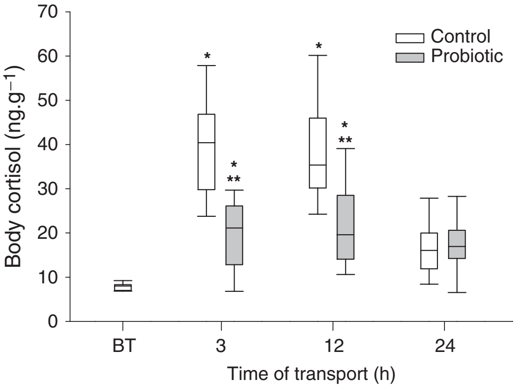

Figure 2 Whole-body cortisol levels during transportation of marbled hatchetfish (*Carnegiella strigata*) with (probiotic treatment) and without addition of Efinol®L in the water (control treatment). BT, before transportation. *Significant differences between BT and all samplings using the Kruskal–Wallis ANOVA on ranks ($P < 0.05$), followed by Dunn's test. **Significant differences between treatments at each sampling time using a *t*-test ($P < 0.05$).

during the transport, reaching 5.82 ± 0.07, 6.29 ± 0.10 and 6.44 ± 0.12 in the probiotic treatment during the 3-, 12- and 24-h samplings. In the control group, the pH reached 5.98 ± 0.06, 6.35 ± 0.07 and 6.72 ± 0.02 in the 3-, 12- and 24-h samplings. Ammonia concentrations were higher in the control treatment in all sampling periods. At 3 h, ammonia concentrations reached 0.40 ± 0.06 and 0.69 ± 0.15 mg L^{-1}, while in the 12-h sample, the levels were confirmed to be 0.94 ± 0.28 and 1.22 ± 0.08 mg L^{-1} for the probiotic and control treatments respectively. In the last water sampling period, ammonia concentrations reached 1.48 ± 0.33 mg L^{-1} in the probiotic group and 1.90 ± 0.10 mg L^{-1} in the control group.

Marbled hatchetfish from both treatments presented very high net Na$^+$ and K$^+$ effluxes after 3 h of transport. After 12 h of transport, net Na$^+$ effluxes tended to lower values, but a significantly lower Na$^+$ efflux was observed only after 24 h of transport in fish exposed to a probiotic (Fig. 1a). Net K$^+$ effluxes in fish of the probiotic treatment decreased significantly throughout the 24 h of transport, reaching values close to zero. Control marbled hatchetfish reduced K$^+$ efflux significantly only after 24 h of transport, but this value was still significantly higher than that of fish exposed to the probiotic (Fig. 1b).

Whole-body cortisol levels were significantly higher in both treatments from the 3- and 12-h samplings when compared with the before transportation (BT), which had a median of 7.82 ng g^{-1} (Fig. 2). The cortisol levels of the control treatment were significantly higher than that of the probiotic treatment at 3 and 12 h of transport. The 24-h median in both treatments was not significantly different when compared with all other samples.

Discussion

There was no mortality at 3 and 12 h of transportation, and at 24 h, mortality was < 1% in both treatments, even using commercial densities. According to Waichman *et al.* (2001), one of the main reasons for ornamental fish mortality during transportation between Barcelos and Manaus is the poor water quality; therefore, the primary reason for the negligible mortality in this work was the adequate water quality during transportation.

At the start of transportation, the oxygen concentration was 5.4 mg L^{-1} in both treatments. However, after 3 h of transportation, the concentrations of dissolved oxygen in the probiotic treatment were higher than that in the control treatment, reaching 3.06 and 2.69 mg L^{-1}, respectively, at 24 h. Waichman *et al.* (2001) reported a similar pattern of oxygen decrease during transportation of marbled hatchetfish, and the concentrations at 24 h also reached 2.6 mg L^{-1}. The results demonstrate that the lower concentrations obtained in the control treatment are not lethal for this fish species. Marbled hatchetfish live in igarapés where fluctuations in oxygen availability and concentrations lower than 1.0 mg L^{-1} are common (Chao & Prada-Pedreros 1995). The lower oxygen concentration in the control treatment may be a result of the excited state of the fish at the start of the experiment.

The pH, conductivity and total ammonia presented a similar pattern, with an increase during transportation and higher values in the control treatment. The increase in pH and conductivity is likely a result of ammonia excretion and ion effluxes. As the ammonia concentration is higher in the control than in the probiotic treatment, other variables should be increased as well. According to Boyd and Massaut (1999), the addition of probiotics composed of *Bacillus* enriched with fermentation products containing extracellular enzymes, such as Efinol®L, is useful in controlling ammonia levels. This affirmation is in agreement with the results of the present work, as the probiotic treatment resulted in lower ammonia concentrations than that in the control treatment. The primary reason for the lower ammonia excretion by the fish in the probiotic treatment is a reduction in metabolic wastes, induced by the action of bacteria in the intestinal tract (Gournier-Chateau, Larpent, Castellanos & Larpent 1994).

The black water from Rio Negro Basin used in the present experiment has very low mineral levels (Mortatti & Probst 2003). In several fish that live in this environment, ion regulation is characterized by high-affinity and high-capacity ion transport systems (Gonzalez *et al.* 1998; Gonzalez & Preest 1999), which allows high rates of Na^+ uptake even in these dilute waters. At the beginning of the transport, marbled hatchetfish presented very high effluxes (loss) of Na^+ and K^+. This was expected because the stress of handling or confinement led to high Na^+ loss in rainbow trout, *Oncorhynchus mykiss* (Walbaum) (McDonald, Cavdek & Ellis 1991), and silver dollar, *Metynnis hypsauchen* (Müller & Troschel) (also Cl^- loss in this species), a fish from Rio Negro (Baldisserotto & Val 2002). Transportation also led to a decrease in serum Na^+ and plasma Cl^- in matrinxã, *Brycon amazonicus* (Spix & Agassiz) (Carneiro & Urbinati 2001), but this loss of ions during transport is not a general rule as pirarucu, *Arapaima gigas* (Schinz), did not show any net ion efflux (Gomes, Chagas, Brinn, Roubach, Coppati & Baldisserotto 2006). The use of a probiotic in the water allowed an equilibrium in Na^+ and K^+ net fluxes in marbled hatchetfish within 24 h (net fluxes close to zero), but the net ion efflux remained high in those maintained under control conditions. As high waterborne ammonia levels stimulated Na^+ efflux in rainbow trout (Twitchen & Eddy 1994), it is possible that the lower waterborne ammonia levels observed in marbled hatchetfish exposed to the probiotic could result in a lower Na^+ efflux. Another explanation for the lower ion loss in marbled hatchetfish exposed to a probiotic could be the higher waterborne Na^+ and K^+ levels (4.4–4.6-fold control levels), which may have reduced the ionic gradient plasma water, decreasing branchial efflux and consequently ion loss. The use of salt (NaCl) in the water reduced ion loss in matrinxã during transportation (Carneiro & Urbinati 2001), but the effective levels were much higher than those in the present experiment (at least 430 00 μmol L^{-1}), and in pirarucu (Gomes *et al.* 2006) and silver catfish, *Rhamdia quelen* (Quoy & Gaimard) (Gomes, Golombieski, Chippari-gomes & Baldisserotto 1999), it only increased osmoregulatory work. Therefore, additional experiments must be performed to determine whether the probiotic effect on ion fluxes of marbled hatchetfish was due to lower ammonia or higher waterborne ion levels or due to any other metabolic effect.

Whole-body cortisol medians of wild unstressed hatchetfish were fivefold lower than those found in the highest concentration (3 h control) during the whole experimental period. There is a significant difference between the control and the probiotic group at the 3- and 12-h samplings, suggesting either a direct influence of Efinol®L on the stress levels or an indirect effect through increased water quality. At 24 h, the cortisol median decreased significantly in both treatments, but was still twofold higher than that of the BT. Unstressed fish had higher cortisol levels than zebrafish, *Danio rerio* (Hamilton), reported by Ramsay, Feist, Varga, Westfield, Kent and Schreck (2006) and also the three-spined stickleback, *Gasterosteus aculeatus aculeatus* L. (Pottinger, Carrick & Yeomans 2002).

The results show that Efinol®L is an efficient probiotic that reduces metabolic waste, cortisol stress and ion effluxes, and consequently, its use during transportation of marbled hatchetfish could improve water quality and reduce osmoregulatory problems related to stress of transport when applied under conditions similar to those found in the present study.

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