Stress responses in juvenile pacu (*Piaractus mesopotamicus*) submitted to repeated air exposure

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ABSTRACT. Pacu juveniles (5.2 ± 1.5 g) were submitted to two one-minute air exposures in a 24h interval, and sampled before the exposure (control) and 5, 15, 30 and 60 min., 24 and 48h afterwards for whole-body cortisol, sodium, potassium and calcium ion concentrations. For the first air exposure, there was a trend of increased cortisol concentration after 15 min., whereas in the second air exposure, the cortisol concentration increased significantly within 5 min. after stress was induced. Sodium ion concentration increased significantly 24h after both air exposures. Potassium concentration presented fluctuations over the experimental period. Calcium ion concentration increased progressively from 5 to 30 min., in both air exposures. The repeated air exposures exacerbated the cortisol response, but they did not affect the recovery ability of pacu over the experimental period. Additionally, the whole-body cortisol measurement might be a reliable indicator of stress, when sampled fish are smaller and blood volumes are very low, making samples inadequate for analysis.

Key words: pacu, *Piaractus mesopotamicus*, stress, air exposure, whole-body cortisol.

RESUMO. Respostas de estresse em juvenis de pacu (*Piaractus mesopotamicus*) submetidos à exposição aérea repetitiva. Juvenis de pacu (5,2 ± 1,5 g) foram submetidos a duas exposições aéreas de um minuto, em intervalo de 24 horas, e amostrados antes da exposição (controle) e 5, 15, 30 e 60 min., 24 e 48 horas depois para análise da concentração corporal de cortisol e dos íons sódio, potássio e cálcio. Na primeira exposição, os peixes apresentaram concentrações de cortisol aumentadas a partir de 15 min., embora não diferissem estatisticamente do controle. Na segunda exposição, a concentração de cortisol aumentou significativamente aos 5 min., retornando às concentrações equivalentes às dos peixes-controle em 30 min.. A concentração do íon sódio aumentou significativamente 24 horas depois das duas exposições aéreas. A concentração do íon potássio apresentou flutuações durante o experimento, enquanto a do cálcio apresentou-se reduzida aos 5 min, aumentando gradativamente até os 30 min., nas duas exposições. A repetição do fenômeno estressor exacerbou a liberação de cortisol, mas não afetou a capacidade de recuperação dos peixes. A análise do cortisol tecidual pode ser considerada um indicador confiável de estresse.

Palavras-chave: pacu, *Piaractus mesopotamicus*, estresse, exposição aérea, cortisol corporal.

Introduction

The handling of fish is a common and unavoidable procedure in fisheries management and aquaculture. This handling can induce severe stress in fish and can result in chemical and biological changes in an attempt to compensate for the challenge imposed upon it, and even mortality or increased susceptibility to disease (Barton and Iwama, 1991; Wendeelar Bonga, 1997). Much work has been done on quantifying the physiological responses to various types of stressors in adult fish (Mazeaud *et al.*, 1977; Barton and Iwama, 1991; Barton, 2002), but little work has been done on young, immature fish (Gomes *et al.*, 1999; Luz and Portella, 2005; King and Berlinsky, 2006; Barcellos *et al.*, 2007).

Reponses to stress-related disturbances in fish are often characterized as primary, secondary, and tertiary. A primary response is the activation of brain centers, which eventually results in the release of cortisol from the steroid-producing cells and of catecholamines from the head kidney chromaffin cells. Secondary responses are defined as the subsequent actions and effects of these hormones at blood and tissue level, and may include a disturbance of the hydromineral and metabolic balance (Barton and Iwama, 1991). Otherwise, there is little information on the physiological response of fish to a persistent stressor and how fish respond when exposed to sequential stressors (Schreck, 2000). How fish respond to single and multiple stressors is obviously important for considering fish health and for environmental risk assessment. Questions related to the importance of the time duration between stressors and the number of stressful events need to be addressed.

Fish handling, including capture and air exposure, is one of the stressors to which fish are submitted during farming. At transport, for instance, fish are submitted to repeated air exposures, initially from being taken out of the tank, and then after depuration when being packed. Air exposure time produced a 50-fold increase in plasma cortisol concentrations in gilthead sea bream (*Sparus aurata*) (Arends *et al.*, 1999) and pacu (*Piaractus mesopotamicus*) (Krieger-Azzolini *et al.*, 1989), and jundiá (*Rhamdia quelen*) (Barcellos *et al.*, 2001) submitted to capture responded with elevated plasma cortisol. The objective of the present study was to assess the stress responses of juvenile specimens of pacu (whole-body levels of cortisol and sodium, potassium and calcium ions) after repeated air exposure.

Material and methods

Experimental design, samplings and measurements

A total of 1,575 fish (5.2 ± 1.5 g) were randomly separated into 21 60 L aquaria (75 fish per aquaria) with constant water and airflow. Fish were submitted to two 1-minute air exposures, spaced 24h apart and sampled before exposure (control) and 5, 15, 30 and 60 min., 24 and 48h after each exposure. At the first time, all fish were suspended in the air, except those in three aquaria that constituted the first control group. Fish of three aquaria were sampled in each turn (four fish per aquaria) and taken out of the experiment in order to avoid accumulated stress in the next sampling. In the second air exposure, the previous procedure was repeated. Before the second air exposure, fish that had been exposed 24h before, from 3 aquaria, were sampled to constitute the second control group. Then fish were sampled using the same procedures and times described previously. Fish were anesthetized (benzocaine, 1 g 20 L^{-1}), frozen in dry ice and stored at -20°C for further whole-body measurement of cortisol, sodium, potassium and calcium. The whole-body cortisol extraction method was based on Jesus *et al.* (1991), with modifications. Cortisol analysis was performed by Radioimmunoassay (RIA, Kit Coat-a-Count from DPC) and results were validated through serial dilutions of the body extracts and verification of parallelism with cortisol standard curve. The minimum detectable limit of the assay was 0.2 ng g^{-1}; the recovery rate was 72.15 ± 4.6% (n = 5); the intra-assay variation was 14.3% (n = 5); and the inter-assay coefficient was 13.1% (n = 5). Sodium, potassium and calcium ions were quantified in a flame spectrophotometer after nitropercloric digestion. In each sampling, 4 fish per aquaria were frozen (2 for cortisol and 2 for ion determination). Water quality was monitored during the experiment. Total ammonia (0.011 ± 0.005 mg L^{-1}), pH (7.16 ± 0.14) and temperature (24 ± 0.4°C) were considered appropriate for tropical fish (Proença and Bittencourt, 1994).

Statistical analyses

The experimental design used was entirely randomized in a 2 x 7 factorial scheme with 2 treatments (repeated air exposures) and 7 sampling times (before the air exposure or control and 5, 15, 30 and 60 min., 24 and 48h) as factors. Results were assessed by means of two-way analysis of variance and means compared through the Tukey test ($p < 0.05$). Statistical analyses were performed using the SAS program (version 8.2, SAS Institute, Cary, NC). Values (n = 6 for each group) are depicted as means ± standard error of the mean (S.E.M).

Results and discussion

In the first air exposure, there was a trend of increased fish whole-body cortisol concentrations (ng g^{-1} of wet body weight) from 15 min. to 24h after the stressor. In the second air exposure, whole-body cortisol increased significantly within 5 min. after stressor, returning to control levels in 30 min. (Figure 1).

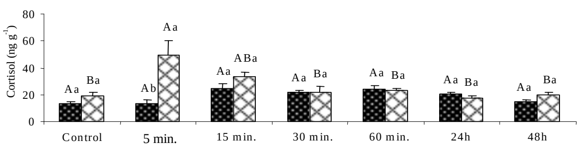

Figure 1. Whole-body cortisol concentration in juvenile pacu after sequential air exposures. (■) first exposure, (☒) second exposure. Different capital letters indicate difference between sampling times in each treatment, and lowercase letters between treatments in each sampling.

Figura 1. *Concentrações de cortisol corporal em juvenis de pacu submetidos à exposições aéreas seqüenciais. (■) primeira exposição (☒) segunda exposição. Letras maiúsculas diferentes indicam diferenças estaticamente significativas entre tempos em cada tratamento e letras minúsculas entre tratamentos em cada amostragem.*

The ion concentrations (mg kg^{-1} of wet body weight) are presented in Figure 2. Sodium concentration in the body showed the same profiles in both air exposures, increasing significantly 24h after the stressor phenomena. Whole-body potassium concentration was lower in the second air exposure, regardless of the sampling, and presented fluctuations over the experimental period. Calcium concentration in the body had similar responses in both air exposures, with reduced values 5 min. after the stressor followed by gradual increases until 30 min. later.

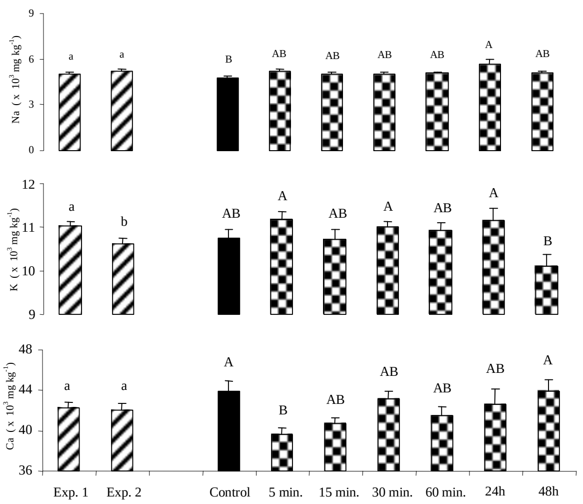

Figure 2. Whole-body sodium (Na^+), potassium (K^+) and calcium (Ca^{+2}) ion concentration in juvenile pacu after sequential air exposures. (▨) air exposures; (▣) sampling times. Different capital letters indicate difference statistically significant between sampling times, and lowercase letters between exposures.

Figura 2. *Concentrações de sódio (Na^+), potássio (K^+) e cálcio (Ca^{+2}) em juvenis de pacu submetidos à exposições aéreas seqüenciais. (▨) exposições aéreas (▣) tempos de amostragem. Letras maiúsculas diferentes indicam diferenças estatisticamente significantes entre tempos de amostragem e letras minúsculas entre exposições.*

The pattern of whole-body cortisol changes following air exposure indicates that juvenile pacu show a typical general stress response (Barton and Iwama, 1991). While whole-body measurement is not directly comparable to plasma measurement, it can be useful when working with small specimens that have very low blood volumes, making it difficult to obtain an adequate sample for analysis. Whole-body levels of many physiological parameters, determined from tissue homogenates, have been used to assess the effects of stressors on fish (Pottinger *et al.*, 2002; King and Berlinsky, 2006). To our knowledge, very little work has been done to investigate stress indicators, such as cortisol in whole-body samples of Brazilian fish species (Barcellos *et al.*, 2007). Gomes *et al.* (1999) measured sodium and potassium in the bodies of jundiá (*R. quelen*) fingerlings, in addition to mortality, to evaluate the stress of transport. Recently, Luz and Portella (2005) evaluated the stress resistance rate of trairao (*Hoplias lacerdae*) larvae and juveniles by exposing specimens to the air on the surface using drying paper and determining the survival after 24h.

Pacu that were exposed to air did not experience mortality, although fish have shown cortisol concentration characteristics of stressed fish (Barcellos *et al.*, 2001), as was shown by increased cortisol in both exposures. In the first exposure, from 15 min. to 24h after the stressor event, fish experienced increases in whole-body cortisol concentrations at levels of 10 ng g^{-1}, in relation to the control values, which can promote a significant impact on body homeostase, although the values were not statistically significant. In the second air exposure, body cortisol increased significantly within 5 min. after the stressor event in levels higher than those obtained 24h before, and recovered to control levels within 30 min., reflecting the accumulated effect of the repeated handlings. Fish are frequently exposed to a series of stressful events in succession. Little is known about how close together in time stressors need to be experienced before they are perceived as one single, perhaps more severe stressor, or how far apart stressors need to be before the experience with the first stressor does not influence the effect of a subsequent stressor (Barton, 2002). Barton *et al.* (1985) demonstrated that rainbow trout (*Salmo gairdneri*) have a cumulative stress response, as measured by elevated plasma cortisol and glucose, to at least three sequential stressors separated by 3-h intervals. The effects of each subsequent stressor were cumulative to those caused by the previous stressor or stressors. However, when chinook salmon (*Onchorhynchus tshawytscha*) were exposed to stressors twice a day sequentially over a period of several weeks, those fish experiencing the stressors had lower cortisol, glucose and lactate responses and more rapid recovery than fish with no prior experience with stress when exposed to subsequent severe handling/crowding for several hours. The length of time between stressors can thus affect the physiological response to a subsequent stressor (Schreck *et al.*, 1995). In pacu, the presentation of the stressor spaced 24h apart showed a rapid cumulative response of cortisol recovering the control condition between 15 and 30 min. To confirm the accuracy of the whole-body measurement of cortisol, the same profile registered in juvenile pacu was described in gilthead sea bream after air exposure (Arends *et al.*, 1999). In addition, elevation of circulating cortisol was registered in pacu (Krieger-Azzolini *et al.*, 1989) and jundiá (Barcellos *et al.*, 2001) after capture.

Many stressors affect the hydromineral balance in fish (Wendelaar Bonga, 1997). Physiological responses to handling and capture for some species of freshwater fish have included decreased concentrations of plasmatic ion sodium (Carmichael *et al.*, 1983; Redding and Schreck, 1983; Carneiro and Urbinati, 2001) and increased concentrations of plasma potassium (Carmichael *et al.*, 1983; Carneiro and Urbinati, 2002) showing sensitivity to stressors. However, a paucity of information exists on the changes of calcium plasma levels during stress (Biswas *et al.*, 2006).

In our experiment, a disturbance of the mineral balance was found in fish stressed by air exposure. The whole-body sodium concentration of pacu showed the same profile in both air exposures, increasing significantly only 24h after exposure. However, this result had no similarity with the findings reported for other freshwater fish (Carmichael *et al.*, 1983; Redding and Schreck, 1983; Carneiro and Urbinati, 2001), which showed decrease in plasma sodium during the stress response. No alteration in carcass sodium levels was registered in *R. quelen* fingerlings after transport (Gomes *et al.*, 1999). The repetition of the air exposure spaced 24h apart did not affect the sodium responses in pacu. The alteration in gill permeability by effect of cathecolamines secreted during the stress (Barton and Iwama, 1991) should facilitate the movement of plasma sodium to the water and decrease the whole-body amount of the ion. It was not observed in our study.

Potassium ion concentration was lower in the second air exposure, regardless of the sampling, and presented fluctuations over the experimental period that did not have apparent relationship with typical stress responses. Increase in serum potassium levels was reported after transport of smallmouth bass (*Micropterus dolomieui*) (Carmichael *et al.*, 1983) and matrinxã (*Brycon cephalus*) (Carneiro and Urbinati, 2002), and may be due to elevated red blood cell fragility provoked by stress, which can result in cell hemolysis during blood processing (Hattingh and Van Pletzen, 1974). Ruane *et al.* (1999) reported the typical plasma coloration caused by red blood cells hemolysis in stressed fish, reinforcing the theory of cell membrane rupture in stressed fish. Conversely, in red sea bream (*Pagrus major*), potassium plasma levels decreased significantly after acute stress and returned to levels indistinguishable from those of control within 72h (Biswas *et al.*, 2006). No alterations in potassium carcass levels were registered in jundiá fingerlings after transport (Gomes *et al.*, 1999). However, the whole-body measurement cannot differentiate levels of substances in specific compartments, and the movement of potassium is between blood cells and plasma. The potassium measurement in whole-body samples might not show differences in the total ion amount if there were no losses to the water.

Calcium ion concentration had similar responses in both air exposures, with reduced values 5 min. after the stressor presentation and gradual increase within 30 min. Calcium has not been usually interpreted as a stress indicator. Although Ca^{2+} is not a major electrolyte in blood plasma (2 mmol L^{-1}) relative to Na^{+} (150 mmol L^{-1}) in freshwater fishes, this ion is essential in many physiological processes, such as control of membrane permeability, activation of muscular contraction, transmission of nervous impulses and bone formation. Ca^{2+} uptake occurs predominantly through the gills, and it is under a tight hormonal control because freshwater fish are often exposed to a constant supply of Ca^{2+} in the water (Flik and Verbost, 1993). Hypocalcaemic factors, such as stanniocalcin and calcitonin, as well as hypercalcemic factors, such as prolactin and somatolactin, have been described (Kaneko and Hirano, 1993; Flik *et al.*, 1995). Additionally, cortisol influences the regulation of calcium uptake, promoting hypercalcaemic effects in fish (Flik and Verbost, 1993). In air-exposed pacu, there was a loss of body calcium 5 min. after air exposure, suggesting calcium transfer from the body to water when cortisol was elevated. In red sea bream, plasma levels of calcium decreased significantly 24 and 48h after acute handling, recovering within 72h (Biswas *et al.*, 2006) in a similar fashion as pacu, in spite of the difference in time after stressor. Future studies will need to focus on the role of calcium during the stress responses, as well as on its regulating mechanism.

Conclusion

The accumulation of repeated air exposures in 24h exacerbated the cortisol response of juvenile pacu soon after the second stressor, but it did not affect the recovery ability of fish over the experimental period. Furthermore, whole-body cortisol measurement proved to be a reliable indicator of stress in small fish, when blood volumes are inadequate for analysis, although other stress indicators investigated are not.

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