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Repeated stressors do not provoke habituation or accumulation of the stress response in the catfish *Rhamdia quelen*

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Fish repeatedly experience stressful situations under experimental and aquaculture conditions, even in their natural habitat. Fish submitted to sequential stressors can exhibit accumulation or habituation on its cortisol response. We posed a central question about the cortisol response profiles after exposure to successive acute stressors of a similar and different nature in *Rhamdia quelen*. We have shown that successive acute stressors delivered with 12-h, 48-h, and 1-week intervals provoked similar cortisol responses in juvenile *R. quelen*, without any habituation or accumulation. The cumulative stress response is more associated to short acute stressors with very short intervals of minutes to hours. In our work, we used an interval as short as 12h, and no cumulative response was found. However, if the length of time between stressors is of a day or week as used in our work the most common and an expected phenomenon is the attenuation of the response. Thus, also, the absence of both accumulation of the stress response and the expected habituation is an intriguing result. Our results show that *R. quelen* does not show habituation or accumulation in its stress responses to repeated stressors, as reported for other fish species.

Os peixes, tanto em condições de cultivo quanto em condições experimentais ou na natureza, podem experimentar situações de estresse. Peixes submetidos a estressores sequenciais podem apresentar acumulação de sua resposta de cortisol com aumento dos níveis desse hormônio em cada resposta sucessiva ou habituação da resposta do cortisol, com redução dos níveis de cortisol em cada resposta sequencial. Abordamos a questão central de como é o perfil de resposta do cortisol de *R. quelen* após a exposição a estressores agudos sucessivos de natureza semelhante e diferente. Os dados mostraram que sucessivos estressores agudos com intervalos de 12 e 48h e de uma semana, provocaram respostas de cortisol semelhantes em juvenis de *R. quelen*, sem qualquer habituação ou acumulação. No presente trabalho usamos intervalos tão curtos quanto 12h e não verificamos qualquer tipo de acumulação da resposta. No entanto, se o intervalo entre estressores forem de um dia ou semana, o fenômeno mais comum e esperado é o da atenuação da resposta. Assim, tanto a ausência de acumulação da resposta ao estresse quanto à ausência da habituação esperada são resultados interessantes. Nossos resultados mostraram que juvenis de *R. quelen* não mostram habituação ou acumulação em suas respostas de estresse a estressores repetidos, como relatado para outras espécies de peixes.

Key words: Cumulative stress, HPI axis, Jundiá, Silver catfish.

Introduction

Fish repeatedly experience stressful situations under experimental and aquaculture conditions, even in its natural habitat. These stressful stimuli are termed stressors, and the response mounted by the organism to cope with these challenges, the stress response; the latter is a complex response that is coordinated, for the most part, by the hypothalamic-pituitary-interrenal (HPI) axis. The end point of activation of this neuroendocrine axis is release of the glucocorticoid cortisol, a steroid hormone that is responsible for several changes aimed at restoration of homeostasis in organisms, which is being challenged by the stressors (as reviewed by Barton & Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002).

Rhamdia quelen (Quoy & Gaimard), the jundiá, a nocturnal catfish species of the family Heptapteridae, also known as the silver catfish, is a commercially important fish in southern South

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America and shares many similarities with other catfish species. Therefore, jundiá may be used as a model for other catfish worldwide. Although its annual production oscillate around 1300 tons, representing less than 0.5% of the Brazilian continental aquaculture, it is in clear expansion (Brasil, 2010), especially in southern Brazil. As stated by Barcellos *et al.* (2004), the more feasible sequence for *R. quelen* culture is hatchery, nursery (from 1-2 to 10 g), and termination (600-800 g). In all phases, several stressful managements were imposed to the fish as sampling for biometrical measurements, sampling for disease verification and size selection (due to cannibalism). Most of these managements are performed in weekly periodicity or alternating with another potential stressful management.

Fish submitted to sequential stressors can exhibit accumulation on its cortisol response, with increased levels of cortisol release in each successive response (Carmichael *et al*., 1983; Flos *et al*., 1988; Maule *et al*., 1988). In contrast, in some fish species like Nile tilapia (Barcellos *et al*., 1999), the sequence of similar stressors provokes a habituation of the cortisol response.

The stress response is mounted to cope with challenges imposed by the stressors and thus restore the organism homeostasis. If this response was exacerbated (accumulated), the effects of the high levels of corticosteroids might be highly deleterious to fish (Barton, 2002). Also, if the response is impaired (attenuated), the changes and responses might be insufficient to totally restore the fish homeostasis, making fish unable to handle the imposed stress (Hontela, 1998).

Thus, we posed a question about the cortisol response profiles after exposure to successive acute stressors of a similar and different nature in *Rhamdia quelen*. We hypothesized that *R. quelen* after successive stressors presented either an (1) accumulation or (2) habituation of its cortisol response. To test these hypotheses, we conducted two simple experiments to evaluate the cortisol response to stress in the following: (1) fish submitted to two similar stressors and a third different stressor at diferent time intervals, and (2) fish submitted to three similar stressors at diferent time intervals.

Material and Methods

To test our hypothesis, we conducted two experiments: "A" and "B" experiments, with three trials each A1, A2, and A3 and B1, B2, and B3. We studied a homogeneous mixed sex group of *R. quelen* consisting of 960 fish (N = 160 per experiment trial) at 90 d of age, with a mean (±S.E.M.) weight of 24.73 ± 0.67 g and total length of 14.11 ± 0.14 cm. All the fish were bred and maintained in 6200-L plastic ponds and fed twice a day with a 32% crude protein diet during the pre-experimental period.

In all experiments, fish were randomly distributed into four 1000-L fiberglass tanks with a blue background and an appropriate number of dark refuges (Barcellos *et al*., 2009), which were filled with 200 L of dechlorinated tap water at a density of 5 g L^{-1}. A previous study has reported that this density is not stressful for *R. quelen* (Barcellos *et al*., 2001).

A schematic of the experimental design of the two experiments is given in Fig. 1. In experiment "A," prestress cortisol levels were measured in individuals from group 1, whereas individuals from groups 2, 3, and 4 were exposed to an acute handling stressor (chasing with a pen net for 60 sec). Cortisol levels were measured in group 2 at five minutes after exposure, according to the procedure reported by Koakoski *et al*. (2012). The cortisol levels were measured in group 3 individuals after they were exposed to the initial stressor and to an additional handling stressor at 12 hours, 48 hours, and 1 week after the first stressor (trials A1, A2, and A3). Fish from group 4 were exposed to both the first and second handling stressors, separated by 12h, 48h, and 1 week; after 12h, 48h and 1 week, respectively, the fish were exposed to a third acute stressor consisting of aerial emersion for 60 sec, which was followed by cortisol measurement. Both handling and aerial emersion causes stress in *R. quelen* resulting in a typical cortisol response (Barcellos *et al*., 2009; Barcellos *et al*., 2011). Additionally, control fish were maintained in the same conditions without any provoked disturbance and were named as control 12h, 48h, and first week; 24h, 96h, and second week; and 36h, 144h, and third week.

Experiment "B" was similar to "A,", except that as the third acute stressor, fish from group 4 were again exposed to handling, before cortisol sampling.

The time intervals were chosen because of the cortisol profiles measured previously in *R. quelen* (Barcellos et al., 2009; Koakoski et al., 2012). We choose for experiments "A1" and "B1" an interval smaller than the 24-h recovery period; for experiments "A2" and "B2," an interval bigger than the recovery period; and for experiments "A3" and "B3," an interval of several days after the cortisol profile recovery.

Because the time course of cortisol response, including the exact moment of the peak values, are well determined in *R. quelen* in these exact experimental conditions (Barcellos *et al*., 2009; Koakoski *et al*., 2012), we evaluated only the cortisol concentration at the peak moment and at the time in which these levels are high (5 min. as determined by Koakoski *et al*., 2012). In both experiments, nine fish were sampled in each sample moment.

To sample fish, buffered MS222 (Finquel®; Argent Chemical Laboratories, Redmons, NE, USA; 300 mg L^{-1}) was applied as anesthetic before withdrawing from each fish 1-2 ml of blood from the caudal vein into heparinized syringes. The collected blood samples were centrifuged for 10 min at 10,000 *g* and were stored at -20°C. Cortisol levels were measured using an enzyme-linked immunosorbent assay (ELISA; EIAgen™ Cortisol Test; BioChem ImmunoSystems). Test specificity was assessed by comparing the standard curve obtained for human cortisol levels with the curve obtained for dilutions of serum samples in PBS (pH 7.4). Using linear regression, we found a highly positive correlation (R^2 = 0.9818) between the curves, yielding interassay and intra-assay coefficients of b variation of 9-12% and 6-9%, respectively.

The Graph Pad InStat 3.00 statistical package (GraphPad Software, San Diego, CA, USA) was used to determine the

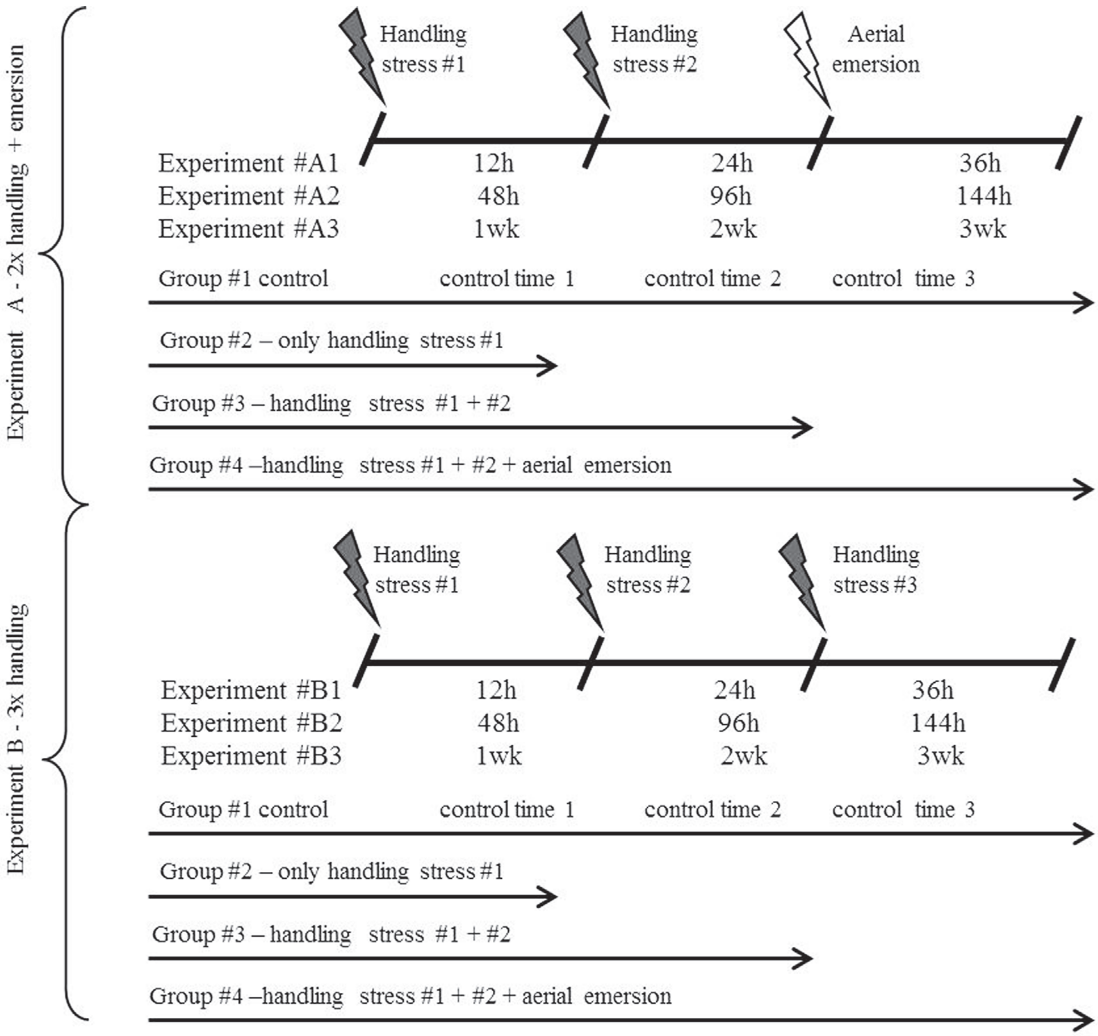

Fig. 1. Schematic representation of the experimental design of both groups of experiments. In experiment "A," *Rhamdia quelen* (Quoy & Gaimard) fingerlings were sequentially exposed to the same stressors twice and to a different stress the third time. In experiment "B," *R. quelen* fingerlings were sequentially exposed as the same stressors 3 times.

mean ± SEM values for each group, whereas two-way analysis of variance (ANOVA), followed by Tukey's test, was used to analyze the cortisol values. A Hartley's test and a Kolmogorov-Smirnov test were used to determine the homogeneity of variance and normality, respectively. A $p < 0.05$ was considered as statistically significant.

Results

For both "A" and "B" experiments, the cortisol values for groups 2, 3, and 4 were higher than the prestress cortisol level for group 1 and also higher than the control values in the 12h, 48h, and first week; 24h, 96h, and second week; and 36h, 144h, and third weeks (Fig. 2).

In a general manner, successive acute stressors at a 12h, 48h, and 1-week intervals provoked similar cortisol responses in juvenile *R. quelen* that were exposed to a different stressor at the third time (experiment "A") and those that were exposed to the same stressor 3 times (experiment "B").

Discussion

Here, we have shown that successive acute stressors delivered with a 12-h, 48-h, and 1-week intervals provoked similar cortisol responses in juvenile *R. quelen*, without any habituation or accumulation, either when the same stressor was repeated 3 times or when the third stressor was different. Our results are intriguing, given that both habituation and accumulation of the stress response to sequential/additional stressors have been reported in the literature.

As reviewed by Barton & Iwama (1991) and Barton (2002), fish can exhibit a cumulative response to repeated stressors.

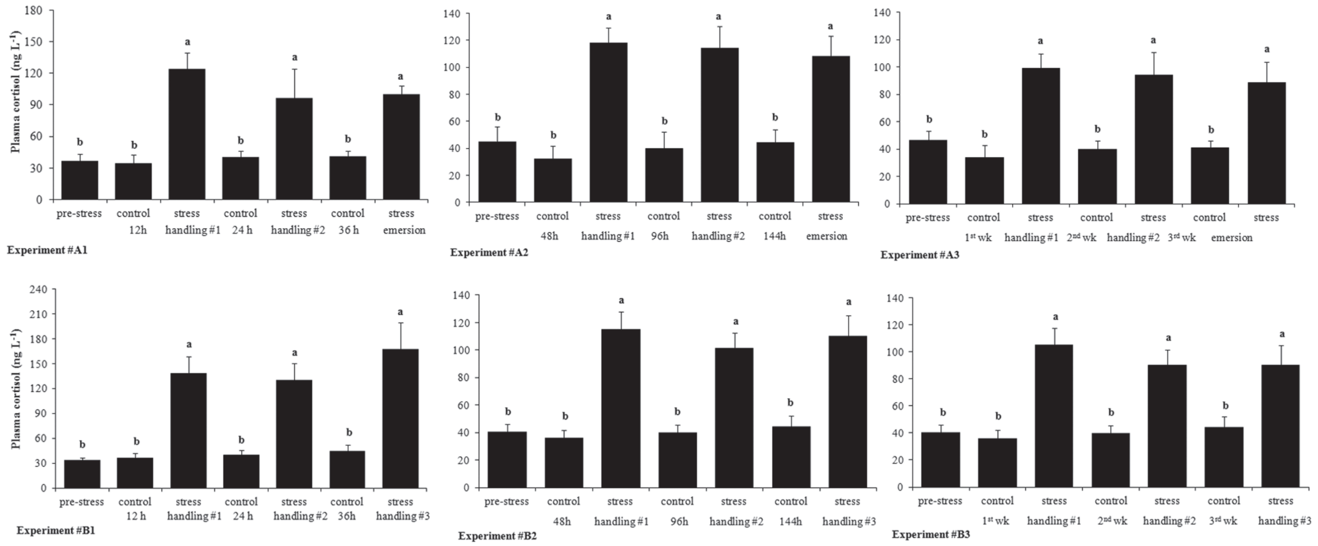

Fig. 2. Plasma cortisol concentrations of *R. quelen* (Quoy & Gaimard) fingerlings exposed to sequential acute stressors. Comparison of responses to two similar stressors and a third different stressor in experiment "A" and comparison of responses to three sequential stressors of the same type in experiment "B." Data are expressed in terms of mean ± S.E.M. values. The different small letters above the histograms indicate statistical differences by ANOVA, followed by Tukey's range test. (n = 8-9).

Barton *et al*. (1986) found that the peak cortisol responses after the final disturbance were cumulative when juvenile Chinook salmon [*Oncorhynchus tshawytscha* (Walbaum)] were exposed to multiple handling stressors with a 3-h interval. Cumulative stress is of importance for aquaculturists because it indicates that, although sublethal disturbances may not appear to be harmful, accumulation of responses over time could be detrimental to individual fish and, thus, to the population (Barton & Iwama, 1991).

Moreover, repeated exposures to mild stressors are also known to desensitize fish and attenuate the neuroendocrine and metabolic responses to exposure to subsequent stressors (Reid *et al*., 1998). For example, Barton *et al*. (1987) subjected juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum), to 1 of 3 different brief handling stressors, once a day for 10 weeks; at the end of that period, their response to acute handling was measured. The plasma cortisol response at this time was approximately half of that observed in naive, previously unstressed fish, indicating possible desensitization of the HPI axis to the repeated disturbances. Modulation of the acute stress response by previous chronic stimuli has also been shown for other teleost fish, including the Nile tilapia *O. niloticus* (Barcellos *et al*., 1999) and the salmonid fish *O. tshawytscha* and *Oncorhynchus kisutch* (Walbaum) (Salonius & Iwama, 1993). However, we have previously shown that an antecedent chronic stress period does not attenuate the posterior acute response in *R. quelen* (Barcellos *et al*., 2006).

The length of time between discrete stressors, the effect of multiple stressors, and the severity of continuous stressors are all important factors that are likely to influence the response in fish (Barton, 2002). The cumulative stress response is more associated to short acute stressors with very short intervals of minutes to hours (Schreck, 2000). We used an interval as short as 12h, and no cumulative response was found. However, if the length of the time between stressors is of a day or week, as used in our work, the most common and expected a phenomenon is the attenuation of the response (Schreck, 2000). Thus, also the absence of accumulation of the stress response as well the absence of the expected habituation is an intriguing result.

We cannot rule that *R. quelen* did not show any habituation because of the relevance of the imposed stressors. Both handling (persecution) and aerial emersion might be interpreted by the fish as high dangerous situation. Also, the short time (36 and 144h and 3 weeks) with three stressor repetition might be a very short time to provoke a habituation of the stress response. Thus, good perspectives for continuing studies are to test the effects of different types of stressors on the accumulation and/or habituation of the stress response and expand the number of repeated stressor as well testing more frequent stressors such as daily stressors.

Understanding the attenuation and accumulation responses to repeated stressors is crucial under both experimental and aquaculture conditions because successive and repeated stressors could affect experimental results or growth performance of stocks. In aquaculture practices, repetition of management practices, such as biometry, partial harvest, and water changes, are commonly required. Our results show that *R. quelen* does not show habituation or accumulation in its stress responses to repeated stressors, in these specific times, as reported for other fish species.

Acknowledgments

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