Is hypoxia a stressor to American bullfrog tadpoles?[1]

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ABSTRACT.- Teixeira P.C., França F.M., Rocha G.C., Antonucci A.M., Ferreira C.M. & Ranzani-Paiva M.J.T. 2014. **Is hypoxia a stressor to American bullfrog tadpoles?** *Pesquisa Veterinária Brasileira 34(4):369-373.* Centro de Aquicultura, Universidade Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. E-mail: pa_co75@yahoo.com.br

The aim of this study was to evaluate alterations to the physiological profile of cortisol in pro-metamorphose phase tadpoles of *Lithobates catesbeianus* exposed to hypoxia stressor in a capture experiment and in a crowding experiment. The capture study was performed by the treatments: stress due to individual capture with a hand net, stress due to batch capture with a hand net and stress due to capture by emptying. Three simultaneous replicates was done witch 12 animals were sampled (6 normoxia - immediately blood collection) and 6 hypoxia - blood collection after 15 min of air exposition) in two collection times with 5 days by intervals. The crowding study was performed by the treatments 1 tadpole L^{-1}, 5 tadpoles L^{-1} and 10 tadpoles L^{-1}. Three simultaneous replicates was done witch 8 animals (4 normoxia and 4 hypoxia) were sampled in the zero moment (ZM) - blood collection before the experiment, 6 animals/treatment (3 normoxia and 3 hypoxia) to 4 and 8 days and 18 animals/treatment (9 normoxia and 9 hypoxia) to 12 days. The average values to plasmatic cortisol varying from 1.7 to 5.1ng mL^{-1} (capture study) and 1.0 to 4.2ng mL^{-1} (crowding study). It concludes that the biomarker tested (cortisol) showed no alterations front of the stressor used. Alternatively, a larger response pattern to these stimuli may have been expressed in another level of an unmeasured hormone (corticosterone). And the bullfrog has great ability to adapt to different management compared to other aquatic organisms, which demonstrates the plasticity of these animals.

INDEX TERMS: Frogculture, American bullfrog, *Lithobates catesbeianus*, tadpole, stress, cortisol.

RESUMO.- [**A hipóxia é um estressor para girinos de rã-touro?**] Avaliou-se o cortisol em girinos de rã-touro (*Lithobates catesbeianus*), no estágio de pró-metamorfose ao mecanismo estressor de hipóxia juntamente com captura (1º experimento) e adensamento (2º experimento). O experimento de captura foi composto pelos tratamentos: Captura individual com puçá, Captura em massa com puçá e Captura por escoamento, com 3 réplicas simultâneas onde 12 organismos foram amostrados (6 normoxia - coleta imediata de sangue e 6 hipoxia - coleta de sangue após 15 min de exposição ao ar) em 2 tempos de coleta com intervalo de 5 dias. O experimento de densidade foi composto pelos tratamentos: 1 girino L^{-1}, 5 girinos L^{-1} e 10 girinos L^{-1}, com 3 réplicas simultâneas onde 8 animais (4 normoxia e 4 hipoxia) foram amostrados no momento zero (MZ) - coleta de sangue anterior ao experimento, 6 animais/tratamento (3 normoxia e 3 hipoxia) para 4 e 8 dias e 18 animais/tratamento (9 normoxia e 9 hipoxia) para 12 dias. Os valores médios para o cortisol plasmático foram de 1,7 a 5,1ng mL^{-1} (Experimento de Captura); e 1,0 a 4,2ng mL^{-1} (Experimento de Densidade). Conclui-se que o marcador biologico de estresse utilizado (cortisol) não foi alterado pelo agente extressor. Alternativamente a resposta a este estímulo pode ser expressa em outro nível hormonal (corticosterona). E a rã-touro apresenta ótima capacidade de se adaptar aos diferentes manejos se comparados a outros organismos aquáticos, o que demonstra a plasticidade destes animais.

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TERMOS DE INDEXAÇÃO: Ranicultura, American bullfrog, *Lithobates catesbeianus*, tadpole, estresse, cortisol.

INTRODUCTION

Many adult fishes, amphibians and reptiles increase their dependence upon aerial oxygen uptake as aquatic oxygen declines, and show a variety of cardiac and respiratory responses that facilitate this transition (Burggren & Wood 1981, Burggren & West 1982).

The great majority of studies on the effects of hypoxia on gas exchange processes in ectothermic vertebrates have been confined to responses to acute hypoxia. Respiratoryhomeostasis during acute hypoxic exposure is usually maintained by increases in the convective flow of air or water through gas exchange organs (Dejours 1981). Acute hypoxia is characterized by the remarkably selective redistribution of blood flow that ensures adequate oxygen supply to vital organs as regional blood flow increases in the brain, respiratory muscles, and liver but decreases in the gastroenteric tract, pancreas, spleen, skin, and limb bones. However, this disturbed blood flow distribution returns to the state of normoxia when the hypoxic state ends (Oka et al. 2007).

Compared with most birds and mammals, lower vertebrates (fishes, amphibians, and reptiles) are tolerant of variable oxygen availability (Bickler & Buck 2007). When faced with hypoxia in the environment, anurans respond by increasing ventilation, heart rate and pulmocutaneous blood flow to maintain O_2 delivery in face of the reduced oxygen availability (Kruhøffer et al. 1987, Wang et al. 1994, Gamperl et al. 1999, Wang et al. 1999). These cardio-respiratory adjustments are, at least in part, due to released vagal tone (Gamperl et al. 1999), but the adrenergic control is largely unknown. It is, nevertheless, well established that the sympathetic nervous system can exert significant cardio-respiratory and metabolic functions during stressful situations and that catecholamines affect cardiac function and vascular resistances in amphibians (Lillo 1979, Herman & Sandoval 1983).

The aim of this study was to investigate the possible alterations in level of cortisol in American bullfrog tadpoles (*Lithobates catesbeianus*) exposed to hypoxia stressor.

MATERIALS AND METHODS

This study was composed by two experiments, a capture experiment and a crowding one using the hypoxia as a stressor in American bullfrog tadpoles (*Lithobates catesbeianus*) in the pro-metamorphosis phase.

In the capture experiment, the animals, acquired from the Frog Farming of Aquacultural Center of São Paulo State University (CAUNESP), Jaboticabal/SP, Brazil (mean weight of 9.05g and mean length of 11.96cm) were transported to Experimental Frog Farming of Aquacultural Center of Agricultural Department of São Paulo State (PRDTAVP), Pindamonhangaba/SP, Brazil, and acclimated for 7 days. They were randomly distributed in polyethylene tanks (500 litters), at a density of 1 tadpole L^{-1} witch in the field were conditioned using an agricultural oven where the surface was covered with polyethylene plastic and the sides with nylon screen. The treatments performed were: individual capture with a hand net (Treatment 1), batch capture with a hand net (Treatment 2) and capture by emptying (Treatment 3), the traditional handling used in Brazil frog farming. Each treatment was carried out in simultaneous triplicate in a random order. The samples were obtained on two sets of collections with an interval of 5 days between them witch 12 individuals were used (6 normoxia - blood collected immediately, and 6 hypoxia – blood collected after 15 min air exposition), totalling 72 animals sampled.

In the crowding experiment, the animals, acquired from a particular propriety (mean weight 10.32g and mean length 11.18cm), were transported to Reference Laboratory Unit for Pathology of Aquatic Organisms at the Fisheries Institute in São Paulo/SP, Brazil, and acclimated for 7 days. They were randomly distributed in aquariums in the laboratory, 20 tadpoles per unit witch the treatments performed were 1 tadpole L^{-1} (Treatment 1 - control group), 5 tadpole L^{-1} (Treatment 2) and 10 tadpole L^{-1} (Treatment 3), with 20, 4 and 2 litters of water respectively. Each treatment was carried out in simultaneous triplicate in a random order. The samples were obtained at ZM (zero moment - blood collected before the experiment), 4, 8 and 12 days. For the ZM, 8 animals were sampled (4 normoxia and 4 hypoxia), for the 4 and 8 days, 6 animals/treatment were collected (3 normoxia and 3 hypoxia) and for the final collection, 18 animals/treatment were sampled (9 normoxia and 9 hypoxia), totalling 98 animals sampled. The water levels were readjusted each time tadpoles were removed from the aquariums so that the density of the remaining animals was not altered.

Time to hypoxia was determined through preliminary tests because according to Bickler & Buck (2007) extended periods of hypoxia probably involve gradual changes in homeostasis resulting in injury. The animals under normoxia condition were removed from the tanks/aquariums, placed in plastic boxes filled with water and immediately taken for blood collection. The animals under hypoxia conditions were removed from tanks/aquariums, placed in humid plastic boxes where they were exposed to air for 15 minutes, and then taken for blood collection.

The animals were fed commercial concentrate containing 45 % crude protein, 6 % crude fibber and 9 % ether extract, once per day in a ratio of 1% of live weight.

In the field, the ambient temperature, relative humidity of the air and physical-chemical parameters of the water (temperature, electrical conductivity, pH, dissolved oxygen) were monitored daily. Total ammonia and nitrite were measured weekly. The water of each tank was continuous flow system. In the laboratory, only the physic-chemical parameters of water (temperature, electric conductivity, pH, dissolved oxygen, ammonia, and nitrite) were monitored daily. The hardness, alkalinity, and total ammonia were monitored every three days. The photoperiod was held constant at 12:12. The mortality was monitored daily in both experiments.

For physiological analyses, an aliquot of blood was obtained from the rupture of the caudal vessel with the aid of disposable needles and heparinized tips, after the application of Lidocaine as a local anaesthetic. In accordance with the circadian rhythm of the animals, blood collections took place in the morning hours (Herman 1992). The animals were subsequently anaesthesiated with benzocaine (1:10) and sacrificed. Blood samples were centrifuged at 1006 x *g* for 10 minutes to obtain plasma witch was frozen on –80°C freezer to posterior cortisol analysis with ELISA (Active - Cortisol EIA DSL10 - Diagnostic System Labs USA).

A descriptive analysis of the variables was done to verify the differences between results of cortisol data among the different treatments. The normality was verified using the D'Agostino-Pearson test, and the homogeneity of variances was verified using the Bartlett test. In the capture experiment a two-way analysis of variance was conducted (both factors fixed), followed by Tukey test. In the crowding experiment, because the small number of

observations and the not normal samples, the treatments were compared using the Kruskal-Wallis non-parametric test followed by the Student-Newman-Keuls test. Statistical tests were performed in the BioEstat 4.0 and Minitab 15, considering significant when p≤0.05 (Zar 1999).

RESULTS AND DISCUSSION

The physic-chemical water parameters results, in both experiments, remained within the standards required to conduct tests with amphibians and were considered good using the standards for farming practices of these aquatic organisms according to Culley Jr (1991). No mortality was registered.

The mean plasma cortisol levels obtained during the capture experiment ranged from 1.7 to 5.1ng mL^{-1} (Table 1); in the crowding ranged from 1.0 to 4.2ng mL^{-1} (Table 2).

Some studies report oxygen availability on circulating catecholamines in amphibians (Boutilier & Lantz 1989, Talbot & Stiffler 1991, Kruhøffer et al. 1987, Gamperl et al. 1999, Andersen et al. 2001). Plasma catecholamine concentrations increase during hypoxia associated with acidosis in *Xenopus* and *Ambystoma* (Boutilier & Lantz 1989, Talbot & Stiffler 1991). Another study report arterial blood gases and plasma catecholamine levels during hypoxia in the toad *Bufo marinus* (Kruhøffer et al. 1987, Gamperl et al. 1999, Andersen et al. 2001).

Catecholamines are the first hormones released after exposure to hypoxia, with the highest levels occurring about 30 seconds after the perception of the stressor. Then, a simultaneous increase of cortisol levels occurs, with the highest concentrations of the hormone being detected 30 minutes after the disturbance in sparid red porgy (Rotllant & Tort 1997). *Oncorhynchus mykiss* submitted to air exposure by 30 sec, the cortisol level show to be high 30 min after the proceeding in manipulated fishes than no manipulated fishes (Sloman et al. 2001). Air exposure produced a 50-fold increase in plasma cortisol concentrations and confinement for up to 24 h induced an initial increase in the plasma cortisol concentration up to eight times of that of controls in gilthead sea bream, *S. aurata*. (Arends et al. 1999). In other species, values can be higher, as in the golden perch, *Macquaria ambigua* (240 ng mL^{-1}) measured after 30 min of netting and confinement stress (Carragher & Rees 1994).

Table 1. Mean values of plasma cortisol (ng mL-1) of *L. catesbeianus* tadpoles exposed to hypoxia in the different collections on the capture experiment

Period	T1		T2		T3	
	Individual capture		Batch capture		Capture by emptying	
	(N)	(H)	(N)	(H)	(N)	(H)
1st	1.7	3.0	3.2	3.8	2.7	2.6
2nd	2.3	3.6	5.1	2.5	3.1	2.1

(N) Normoxia = immediate blood collection, (H) hypoxia = blood collected after 15 minutes of air exposure.

Table 2. Median values of plasma cortisol (ng mL-1) of Lithobates catesbeianus tadpoles exposed to hypoxia in the different days of collection on the crowding experiment

Period (days)	T1		T2		T3	
	1 tadpole L-1		5 tadpoles L-1		10 tadpoles L-1	
	(N)	(H)	(N)	(H)	(N)	(H)
0	1.0	2.3	1.0	2.3	1.0	2.3
4	1.8	2.8	1.0	3.9	1.6	4.2
8	2.6	1.5	2.5	2.3	1.5	1.6
12	2.5	2.8	2.7	1.5	2.2	2.1

(N) Normoxia = immediate blood collection, (H) hypoxia = blood collected after 15 minutes of air exposure.

Alterations resulting from the tested stressor stimuli, hypoxia, were not consistent with the classic stress response model; that is, an increase in glucocorticoid levels after exposure to a stressor stimulus.

According to Moberg (2000), Wada (2008), the mechanism related to the control of cortisol secretion is a complex process involving Adenocorticotropic Hormone (ACTH), which highlights the finding, that cortisol does not act alone. Specifically in the premetamorphosis and prometamorphosis stages, the synthesis of corticosteroids varies depending on the presented challenge (stressor stimulus) and the organism's life cycle (Belden et al. 2003, Crespi & Denver 2004a, b, 2005). Plasmatic corticoids levels in the larval phase of most amphibian species show to be low during premetamorphosis, increasing during prometamorphosis and rise to a peak in the clímax of metamorphose in *R. catesbeiana* (Jaffe 1981, Krug et al. 1983, Kikuyama, Suzuki & Iwamuro 1986); in *X. laevis* (Jolivet-Jaudet & Leloup-Hatey 1984); in *Ambystoma tigrinum* (Carr & Norris 1988) and in *R. pipiens* (Glennemeier & Denver 2002a). The animals of both experiments showed low level of cortisol after stress stimulus, similar to basal one of some fishes, even in prometamorphosis phase.

According to Fritsche & Burggren (1996) mild hypoxia is not necessarily detrimental to development, because compensating tissue level responses are evident.

Tadpoles, in fact, live entirely outside the water for considerable lengths of time (Wassersug & Heyer 1983). In addition to having multiple sites for gas exchange and mastering the complexities of managing multiple exchange sites (lungs, gills, skin), the amphibious nature of most species means potentially altering gas exchange strategies on a minute-by-minute basis for the transition from air emersion to water immersion or vice versa (Wang et al. 2004). This 'plasticity' of tissues and even whole organs, which usually manifests itself only during the normal metamorphic process, apparently can be stimulated by environmental factors unrelated to development, such as hypoxic exposure, to produce morphological adjustments in respiratory structures which facilitate gas exchange under sub-optimum conditions (Burggren & Mwalukoma 1983).

In tadpoles of ranid frogs, the gills and other buccopharyngeal surfaces account for no more than 10-40% of total O_2 uptake (Burggren & West 1982, West & Burggren,1982, Burggren, Feder & Pinder 1983). In *R. catesbeiana* tadpoles of developmental stages IV-XIX, about 60 % of oxygen uptake occurs *via* the skin, while this falls to only 20% in the adult bullfrog (Burggren & West 1982). This may result in part from the changing ventilation/perfusion relationships of the cutaneous and pulmonary vascular bed. The skin accounts for the majority of respiratory capillari-

zation (Saint-Aubain 1982), is thin, and is the predominant route of O_2 uptake in airbreathing tadpoles in normoxic water (Burggren & West 1982, West & Burggren 1982, Burggren, Feder & Pinder 1983). Moreover, even though cutaneous gas exchange in amphibians is diffusion-limited along any given capillary (Piiper 1982), tadpoles can augment cutaneous exchange by perfusing additional capillaries or by increasing cutaneous capillarization and thinning the skin (Burggren & Pinder 1982). Lungs are functional early in development witch tadpoles of all stages surface occasionally to breathe air and routinely have gas-filled lungs (Just et al. 1973, Ultsch det al. 1998). According to Burggren & West (1982) and Feder (1983a) tadpoles of most anurans have well-developed lungs and breathe air regularly.

In addition, the partitioning of gas exchange in bullfrog tadpoles among these exchangers and between air and water depends on a number of factors, including body size, respiratory gas concentrations in the water, temperature, stage of development, rate of oxygen consumption (VO_2), and microhabitat (Noland & Ultsch 1981, Burggren & Doyle 1986, Burggren & Infantino 1994, Ultsch et al. 1998). According to Feder (1981, 1982), the effects of mass, trophic state, time of day, feeding and stress were considered as factors influencing VO_2.

So in fact no significant alterations were observed in the cortisol level front of the hypoxia in capture and crowding experiments. The tadpoles of both experiments certainly breathed air by lungs/skin when they were submitted to 15 minutes hypoxia so this mechanism did not represent stressful to elevate the cortisol compared to fishes submitted to the same stressor. Alternatively, a larger response pattern to these stimuli may have been expressed in another level of an unmeasured hormone (corticosterone). Additional studies are necessary to complement the information about stress physiology and its biomarkers for *Lithobates catesbeianus,* especially for intensive rearing systems.

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