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Effects of Mercury Chloride ($HgCl_2$) on *Betta Splendens* Aggressive Display

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Mercury chloride ($HgCl_2$) is a toxic mercury salt and a major pollutant, that can be found in soil, water and air, with influences on behavior, physiology and adaptation to the environment. In this study two experiments were designed to examine interactions and effects of $HgCl_2$ on some behavioral patterns of Siamese fighting fish (*Betta splendens*). In the first experiment we tested the effect of a progressive dose (five 0.04 mg) on aggressive display with exposure to a mirror, whereas in the second experiment we tested the effect of an acute dose (0.2 mg) on the aggressive display with exposure to a mirror. The experiments were performed on 5 consecutive sessions at intervals of 18 hours between sessions. Differences of performance were shown by subjects in the acute and progressive treatments when compared with a control treatment in the majority of behaviors evaluated, namely Floating, Slow Swimming, Wavy Swimming, Emerging, Bend, Square Move and Motor Display Components. Acute treatment was different from control only on Show Body, while the progressive group differed on Resting, Horizontal Display and Appropriate Display Components. Differences between Correlate Display Components and Total were also shown. Both the acute and progressive contamination with $HgCl_2$ decrease the motor activity in the aggressive display, mirror-image test of *Betta splendens*, mainly on the progressive dose. This implies an impairment on feeding behavior, predator avoidance, reproductive behavior, mate choice and territoriality. These results suggest that in this fish species, the progressive dose has a greater effect on behavior in general and that both the acute and progressive contamination with mercury chloride affect many other aspects of behavior.

Keywords: mercury chloride, Betta splendens, aggressive display, aggressiveness.

El cloruro de mercurio ($HgCl_2$) es una sal de mercurio tóxica y un contaminante importante, que se puede encontrar en el suelo, agua y aire, y que influye en el comportamiento, la fisiología y la adaptación al medio ambiente. En este estudio, dos experimentos fueron diseñados para examinar las interacciones y los efectos del $HgCl_2$ en algunos patrones de comportamiento de peces luchadores siameses (Betta splendens). En el primer experimento se evaluó el efecto de una dosis progresiva (cinco 0,04 mg) en la exhibición agresiva con exposición a un espejo, mientras que en el segundo experimento se evaluó el efecto de una dosis aguda (0,2 mg) en la exhibición agresiva con exposición a un espejo. Los experimentos se realizaron en 5 sesiones consecutivas a intervalos de 18 horas entre sesiones. Se muestran diferencias de rendimiento por los sujetos en los tratamientos agudo y progresivas en comparación con un tratamiento de control en la mayoría de las conductas evaluadas, es decir, Flotación, Nado lento, Nado ondulado, Emergente, Doblado, Movimiento cuadrado y componentes de exhibición motora. El tratamiento agudo difiere del control sólo en Mostrar cuerpo, mientras que el grupo progresivo difiere en Reposo, Exhibición horizontal y en Componentes adecuados de exhibición. También se muestran las diferencias entre Correlación entre los componentes de exhibición y Total. Tanto la contaminación aguda como progresiva con $HgCl_2$ disminuye la actividad motora en la exhibición agresiva en la prueba de imagen-espejo de Betta splendens, principalmente de la dosis progresiva. Esto implica un deterioro en el comportamiento de alimentación, de evitación a los depredadores, en el comportamiento reproductivo, la elección de pareja y la territorialidad. Estos resultados sugieren que en esta especie de pez, la dosis progresiva tiene un efecto mayor en el comportamiento en general, y que tanto la contaminación aguda como la progresiva con cloruro de mercurio afecta a muchos otros aspectos del comportamiento.

Palabras clave: cloruro de mercurio, Betta splendens, exhibición agresiva, agresividad.

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Mercury has great potential for bioaccumulation in aquatic environments (Azevedo, 2003), with the hydric and trophic exposition probably being the major route of mercury accumulation in fish (Harris & Bodaly, 1998), that are common in our diet. The primary source of human exposure to mercury comes from consumption of fish contaminated with methylmercury (MeHg) (Newland, Paletz, Donlin, & Banna, 2006). Mercury can also reach humans by direct contact or water consumption. The long retention times of mercury in fish have been well established (Niimi, 1987). Bloom (1992) tested a number of species of wild-caught fish and marine invertebrates using ultraclean techniques, and found that in virtually all (> 95%) mercury was present in the form of MeHg (Niimi, 1987; Bloom, 1992; Smith & Weiss, 1997). However, mercury salts are the most toxic and irritating form of this metal. Mercury chloride ($HgCl_2$) is very soluble in water, and has previously widely been used as an antiseptic an even for such purposes as suicide (Gutierrez, 2002).

According to Yanagisawa (1998), The Hg^{2+} provenient from $HgCl_2$ forms a complex with sulfhydryl-containing ligands such as albumin and glutathione. According to Gutierrez (2002), mercury binds with different ligands of physiological importance, such as phosphoryl, carboxyl, amine and amide groups which are present in many kinds of cells.

"The diversity of therapeutic and toxic roles of mercury is associated with the chemical substituents that affect solubility, dissociation, relative affinity for various cellular receptors, distribution and excretion" (Gutierrez, 2002).

The author (Gutierrez, 2002) indicates that Mercury compounds act on cerebellar granule cells, and on receptors of gamma-aminobutyric acid affecting neurotransmission. Moreover, the inorganic mercury decreases the activity of the enzyme superoxide dismutase and modifies the activity of Glutathione peroxidase on cerebellum and brain stem, when administered for 7 days, therefore, the author concludes that the oxidative stress should contribute to the development of neurodegenerative disorders caused by mercury poisoning.

The visual system is also affected by mercury toxicity. There are studies involving long-term occupational exposure to Hg-vapor, showing persistent color vision impairments (Santana et al., 2010), non-reversible contrast sensitivity impairment (Costa, Tomaza, de Souza, Silveira, & Ventura, 2008), a widespread reduction of sensitivity in both visual fields (Barboni et al., 2008) and long-term effects on information processing and psychomotor function, with increased depression and anxiety also possibly influenced by psychosocial factors (Zachi, Ventura, Faria, & Taub, 2007).

Mercury is easily bonded in contact with other metals such as gold, silver and tin. It is used and released indiscriminately into the environment due to gold mining and related activities, causing contamination in humans and other animals (Hartman, 1995). The burning of fossil fuels is also a source of mercury. Alkali industries, electrical equipment, paints and cellulose are the largest users of mercury, accounting for 55% of total consumption. Mercury has been used in some agriculture products, mainly in fungicides and has a wide variety of uses, such as medicine, dental and military applications and in batteries. Although the industrial use of mercury has been reduced due to stricter regulation, high concentrations are still present in sediments associated with its industrial application (Klaassen, Amdur, & Doull, 1986).

Mercury binds to the microorganism's cell membrane and, according to Boening (2000), there are several natural ways organisms and microorganisms can contain or cancel the Hg, namely: (1) Efflux pumps that remove the ion cell, (2) Enzymatic reduction of metal to less toxic elemental form, (3) Chelation polymers enzyme (i.e., metallothionein), (4) Mercury binding to the cell surface, (5) Precipitation of organic insoluble complexes (usually sulfides and oxides) on the cell surface, and (6) Biomethylation with subsequent transport across the cellular membrane by diffusion. This latter mechanism makes mercury more toxic among the higher organisms.

Although fish in natural populations may carry high body concentrations of both organic and inorganic mercury, the effects of this divalent metal in lower vertebrates are poorly understood (MacDougal, Johnson, & Burnet, 1996). However, studies with methylmercury (MeHg) have shown that poisoning causes behavioral changes that correspond in topography to the fish responses to predators (Smith & Weis, 1997), feeding behavior (Fjeld, Haugen, & Vøllestad, 1998), as well as the anxiety responses of rats and fishes of the *Danio rerio* species (Gouveia et al., 2003). There are also effects on aggression, motility and emotionality, an effect that could be caused by alterations in the mono-amino-oxidase systems (Gouveia, Oliveira, Romão, Brito, & Ventura, 2007).

The methylmercury (MeHg) neurotoxicology in sub-lethal doses is also interesting from an ecological point of view. This substance produces changes that have a large effect on the reproductive capacity and survival of animals in the natural environment. It also has effects on development, and on the interruption of cortical structures formation, including the bias of neurotransmitters such as monoamines and gamma-aminobutyric acid (GABA), which are particularly sensitive to MeHg (Newland et al., 2006). The MeHg is harmful to the nervous system and its effects on behavior are only partly understood as long-term changes due to its accumulation in the body. Moreover, Reed, Paletz, and Newland (2006) proposed that the effects of MeHg are mainly characterized by disruptions in the relationship between the response and its consequences, the role of the stimuli control processes (discrimination, memory) being relatively minimal.

In fish, display tests are often utilized to quantify levels of anxiety and aggression, and studies using these tests have

Table 1
Designation and description of the 12 behavioral patterns measured together with the three behavioral categories used and the Total behaviors

		Display Behaviors	
Categories	Abrev.	Behavior	Explanation
Appropriate Display Components (ADC)	HD	Horizontal Display	Horizontal axis movement, with open operculum and with pectoral, dorsal and caudal fins opened.
	VD	Horizontal Display	Vertical axis movement, with open operculum and with pectoral, dorsal and caudal fins opened.
Motor Display Components (MDC)	OP	Open Operculum	Operculum opened, without HD or VD situation.
	SS	Slow Swimming	Slow movement, vertical or horizontal, without HD or VD, using caudal fins.
	WS	Wavy Swimming	Fast movement, vertical or horizantal, without HD or VD, using caudal fins.
Correlate Display Components (CDC)	F	Floating	None open fin or movement.
	R	Resting	Without movement, lying on the bottom of the aquarium and with none open fin or movement.
	E	Emerging	Emerge to the surface and swallow air.
	SM	Square Move	Characterized by a fast movement in a square shape followed by a charge movement toward the mirror.
	C	Charge	Fast movement toward the mirror in HD or VD situation.
	SB	Show Body	Undulated movement in front of the mirror with HD or VD situation.
	B	Bend	Diagonal position (circa 45°) in front of the mirror, with or without movement, and without HD or VD situation.
Total Behaviors (TOTAL)	T	Total	The sum of all behaviors emitted in each group.

demonstrated that fish poisoned with lead exhibit a decrease in learning, long-term memory formation and a high frequency of behaviors related to offensive aggression, possibly resulting from the dopamine reuptake inhibition (Santos, 2009). However, there are no studies investigating the effects of mercury chloride on mirror-image display tests.

The *Betta splendens*

The aggressive/reproductive display of the Siamese fighting fish (*Betta splendens*), is a species-specific agonistic sequence that may be separated into appetitive, mating, and post-mating components (Klein, Figler, & Peeke, 1976). In the case of *Betta splendens*, the appetitive components that correspond to the display have been extensively studied (Gouveia et al., 2007). These appetitive components are characterized by specific behaviors, such as saturation of body color, erection of the opercles or gill cover, orientation and movement characteristics (Simpson, 1968). The mating-related components include biting, jaw locking between opponents and striking with the tail (Simpson, 1968). An alteration in one of the appetitive components predictably alters the mating components (Klein et al., 1976; Bronstein, 1985). The display is very prominent and Bronstein (1980, 1981, 1982) suggested that this is an agonistic and reproductive strategy typical of many teleosts using external fertilization in relation to the body of the female of the species (see also Simpson, 1968; Gouveia et al., 2007).

The display response should be elicited by placing a member of the same species in the same (or another) aquarium, or by the use of a mirror or subject model (Meliska et al., 1980). The vigor with which animals present their display, defined by the duration and frequency of the demonstration, is a reliable predictor of the animal's performance in a real combat situation (Evans, 1985) and in situations in which dominance is established (Gouveia et al., 2007).

The present study used the same ethogram as that utilized by Gouveia et al. (2007), which presents 12 behavioral patterns of the Siamese fighting fish (see table 1). These patterns were used as a measure for evaluating behavioral changes caused by mercury chloride.

The purpose of this study was to submit subjects poisoned with mercury chloride via intraperitoneal injections to behavioral tests, in order to examine the consequences of its toxicity on the agonistic species-specific display as shown in response to the mirror-image test.

Method

Participants

Nineteen adult males, blue color Siamese fighting fish were acquired from a single commercial source (Neon Aquarius, Belém, Brazil) and only experimentally naïve fish were utilized in the experiments

Apparatus

The subjects were isolated from each other in individual cylindrical tanks with a capacity of 700 ml. (radius 3 cm and height 16 cm), with light exposure controlled, 12/12 h light/dark, resulting in a regular circadian cycle which started at 6:30 a.m. The average pH was 8.0 and average temperature was held constant at 22 ºC.

The subject´s housing period lasted 15 days, in order to allow the fish to become acclimated to the laboratory environment and to control for possible diseases. Food was offered once a day between 8:30 and 10:30 a.m. Three glass aquaria (13 x 10 x 8 cm) were used as experimental aquaria. Each of them was provided with a mirror (14 x 9 cm)

Procedure

The 19 subjects were randomly divided into three groups: one group for acute poisoning ($n = 6$), one group for progressive contamination ($n = 6$), and a third group of 7 subjects served as control group.

The two infected groups were subjected to intraperitoneal injection of mercuric chloride. For the acute group, receiving 0.2 mg of mercuric chloride, the content of the solution used was 0.4 mg $HgCl_2$/ml, with a dose of 0.05 ml per fish, applied at once during the first day. For the progressive dose group, which received only 0.04 mg per day for five days, the preparation was 0.08 mg of $HgCl_2$, with a dose of 0.05 ml per fish. It was necessary to use another concentration to facilitate the injection of the solution. The control group received no treatment.

The fish were poisoned in the afternoon (between 14:00 and 15:00 hours). After 24 hours of contamination, the subjects were placed individually in the experimental aquaria equipped with a mirror close to one of their sides. This mirror was first covered by a white paper to prevent the fish from viewing the mirror before the end of a five minutes habituation period in the apparatus. After five minutes the paper was removed, and the behavior of the fish was recorded with the video camera over a five minutes period. Three fish were recorded at a time in their aquaria. The procedure was repeated for five days. The procedure for contamination of the group that was progressively treated was repeated during a five day period, whereas the acute group was poisoned only during the first day.

The seven subjects in the control group were held 15 days of housing and at the end were recorded in the experimental aquaria for 5 days to have a baseline for the acute and progressive groups. The videos were analyzed a posteriori and the behaviors exhibited by the fish were described as aggressive display components in the ethogram of the species. Then, the behaviors were transcribed with the help of the Etholog 2.2 software Ottoni (2000).

Statistical Analysis

Frequencies of occurrence of each behavior were tested for normality using the Kolmogorov-Smirnov test. For parametric data, a two-way ANOVA (Day and Group) was used. This was followed by the Tukey's HSD post hoc test. For non-parametric data a Kruskal-Wallis analysis of variance was used, followed by Dunn´s multiple comparison test. A p value of ≤ 05 indicated statistical significance and all analyses were performed using SigmaStat 3.1 software.

Results

The Kolmogorov-Smirnov test indicated that only the Total category data matched the pattern expected for a normal distribution, therefore, the only category analyzed by ANOVA was Total behaviors. Kruskal-Wallis analysis of variance indicated that the variable Day has not statistical significance except for Wavy Swimming [$H(4) = 10.467$, $p = .033$] on day 3 versus days 1,2,4,5 and day 5 versus day 1 on the control group; Vertical Display [$H(4) = 9.908$, $p = .042$] between all days; and ADC [$H(4) = 10.413$, $p = .034$] and CDC [$H(4) = 10.089$, $p = .039$] on day 2 versus day 5 in the progressive group. Hence only the variable Group was considered in this analysis.

The Between-Groups Analysis showed significant differences for the following behaviors: Floating [$H(2) = 45.878$, $p = .001$], Resting [$H(2) = 19.177$, $p = .001$], Slow Swimming [$H(2) = 16.175$, $p = .001$], Wavy Swimming [$H(2) = 49.612$, $p = .001$], Horizontal Display [$H(2) = 13.151$, $p = .001$], Emerging [$H(2) = 35.738$, $p = .001$], Show Body [$H(2) = 12.559$, $p = .002$], Bend [$H(2) = 32.236$, $p = .001$], and Square Move [$H(2) = 20.329$, $p = .001$]. However, the analysis revealed no significant differences between groups in Vertical Display [$H(2) = 5.448$, $p = .066$], Operculum [$H(2) = 4.976$, $p = .083$], and Charge [$H(2) = 4.821$, $p = .090$] (see figure 1).

The Dunn's multiple comparison test showed significant differences ($p \leq .001$) between control and acute treatments

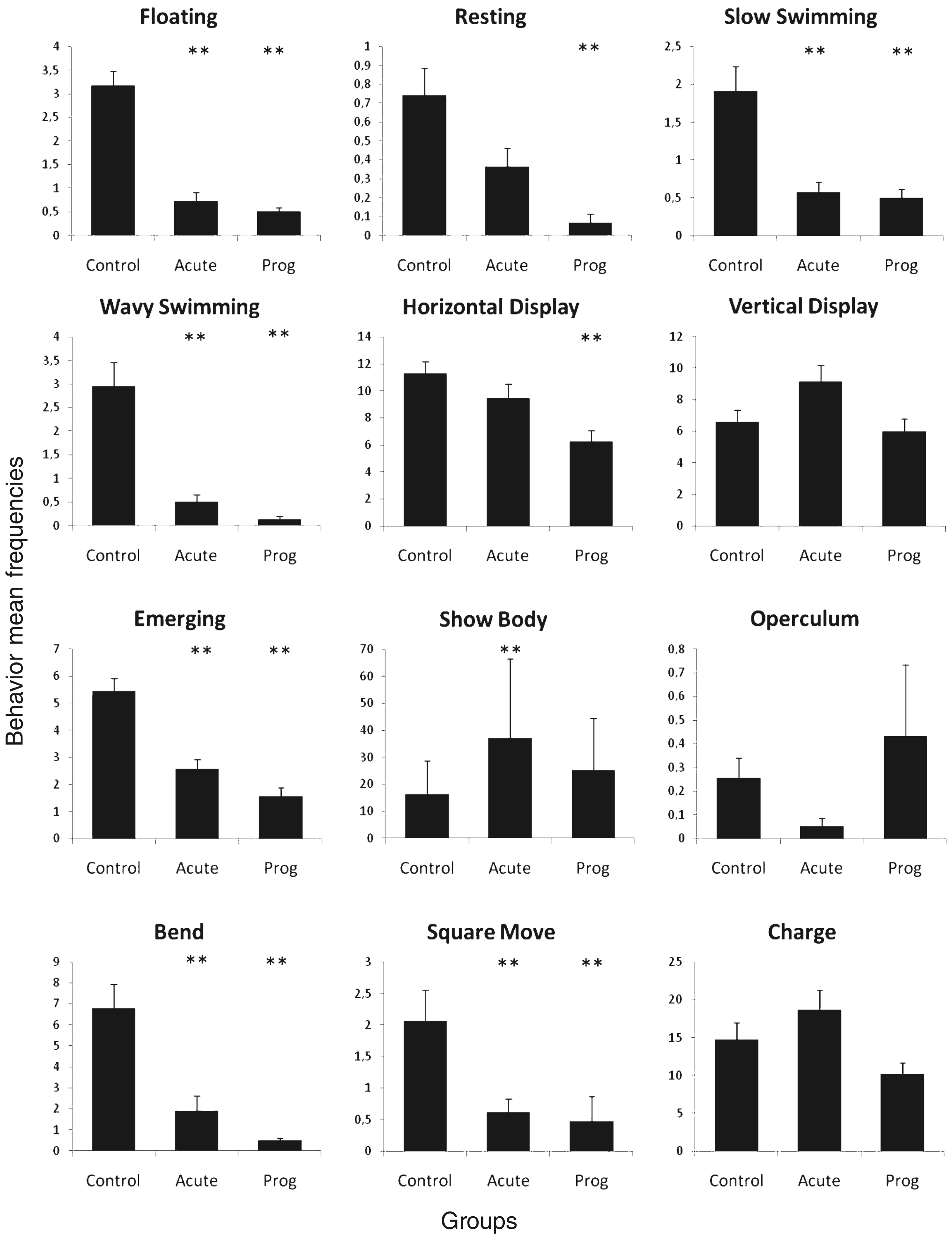

Figure 1. Mean frequency (± standard error) of all behaviors measured in the progressive, acute and control group. **= differs from control with $p \leq .001$.

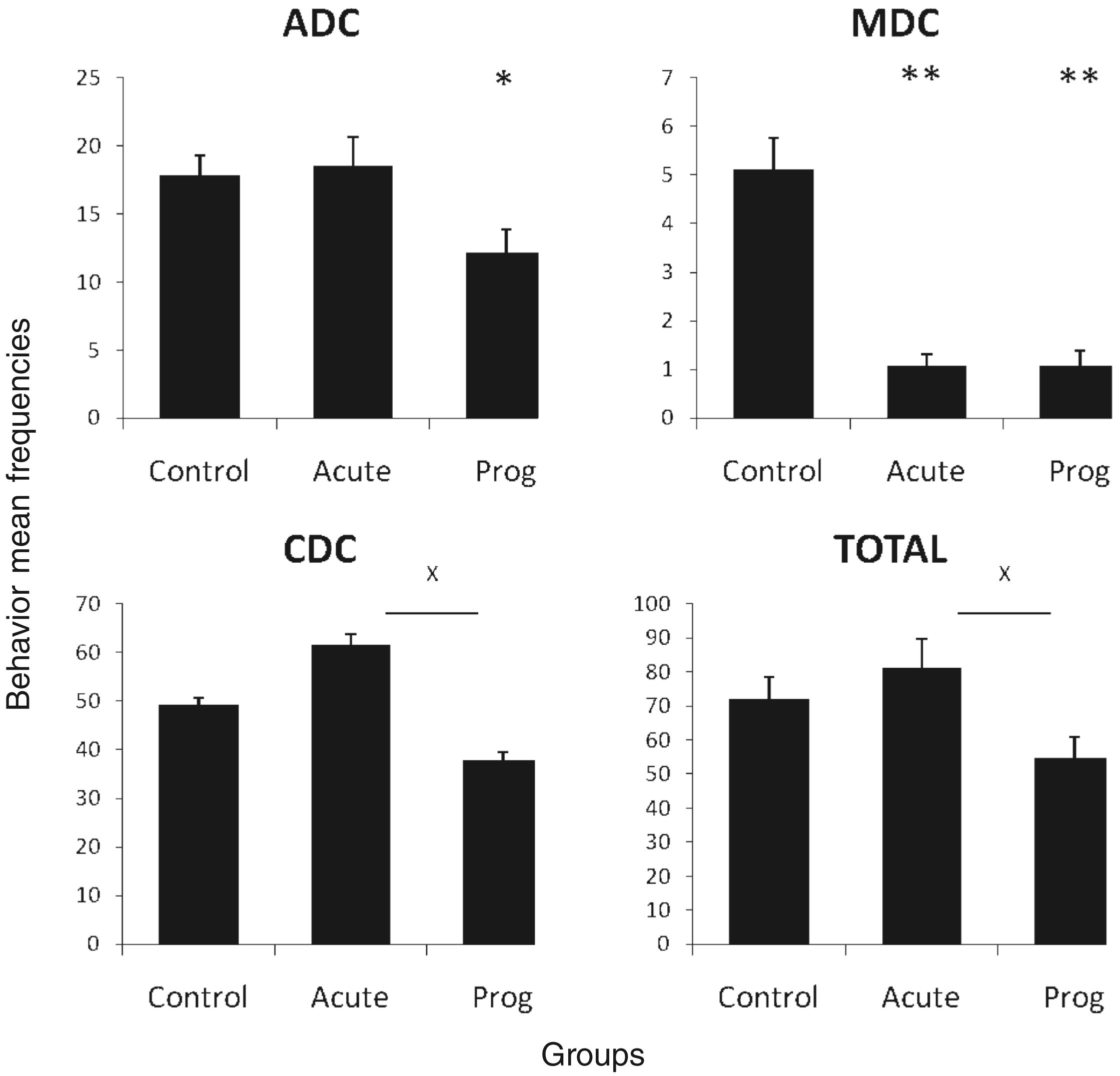

Figure 2. Mean frequency (± standard error) of all categories of behavior in the progressive, acute and control groups. **= differs from control with $p \leq .001$; * = differs from control with $p < .05$; _X_ = difference between acute and progressive groups with $p < .05$.

and between control and progressive treatments in Floating behavior, Slow Swimming, Wavy Swimming, Emerging, Bend, and Square Move. In Resting behavior and Horizontal display a significant difference was only found between control and progressive treatments ($p \leq .001$), and in Show Body significant differences were found only between control and acute treatments ($p \leq .001$) (see figure 1).

When grouping individual behaviors, and considering categories of behavior as Appropriate Display Components (ADC), Motor Display Components (MDC) and Correlate Display Components (CDC) (figure 2), the analysis revealed significant differences among groups in all three behavioral categories: ADC [$H(2) = 7.407$, $p = .025$], MDC [$H(2) = 46.342$, $p = .001$] and CDC [$H(2) = 6.510$, $p = .039$]. The Dunn test showed significant differences between control and progressive in ADC ($p = .025$), between control and progressive and between control and acute in MDC ($p = .001$), and between the acute treatment and the progressive treatment in CDC ($p = .039$). ANOVA of the Total category indicated statistical significance of the Group factor [$F(2, 80) = 3{,}720$, $p = .029$] with significant differences between acute and progressive groups ($p < .05$, Tukey HSD test).

In sum, a remarkable reduction in most of the behaviors was observed when the experimental groups were compared with the control group (see figure 1 and figure 2). In both acute and progressive groups, the behaviors Floating, Slow Swimming, Wavy Swimming, Emerging, Bend, Square Move, and MDC were noticeably lower than the control group, while Resting, Horizontal Display and ADC behaviors had a decrease solely on the progressive group. On the other hand, Show Body showed a significant increase only in the acute group, whereas in the CDC and Total categories clear differences existed between the acute and progressive groups.

Discussion

The present results show that mercury chloride produces a marked global reduction in behaviors whether the treatment was administered in either acute or progressive doses in *Betta splendens*. The decrease was generally greater in the progressive group, which suggests a dose-dependent effect of mercury chloride in this group. Differences in Correlate Display Components (CDC) were found between the acute and progressive groups, which may be attributed to the high levels of the Show body behavior exhibited by the acute group. In this group a slight increase in the frequency of Vertical Display and Charge, together with a significant increase in Show Body behavior was also found relative to control group. These findings suggests that mercury chloride has an effect on components of the aggressive behavior, since Show Body and Vertical Display are typically defensive aggression displays, whereas Charge is related to offensive aggressive display.

However, the decrease in *Betta*´s fish movements in the present mercury poisoning situation may be due to several factors. Contamination by Hg seems to cause a disruption in the relationship between stimulus and response, which can be illustrated in the relationship predator-prey, that requires reflexive and operant responses. Thus, according to Kania and O'Hara (1974), quoted in Smith and Weiss, (1997) the "Mosquitofish" (*Gambusia affinis*) exposed to mercuric chloride becomes more vulnerable to predation by largemouth bass (*Micropterus salmoides*). Likewise, Reed and Newland (2007), stated that contamination by Hg also increases perseverative behaviors, perhaps due to an insensitivity to contingencies, a factor found in their experiment with rats indicating that when the animals were infected with mercury, a quicker acquisition of fixed ratio bar pressing was found. Giménez-Llort et al. (2001) showed that methylmercury seems to decrease sensitivity to reinforcing events, and this in turn may act to delay the behavior changes in situations that require choice, even when ongoing-choice changes are involved.

Also, is possible that *Betta* fish contaminated with Hg decrease their locomotion due to disruption in the stimulus and response relationship, which influences the display elicitation by reference to their own stimulus image in the mirror. This possibility was supported by results of experiments that indicate the persistence of perseverative behavior, due to contingency insensitivity (Reed & Newland, 2007). The above mentioned choice requirement can also be an important factor in the decrease in locomotion, because it can influence the execution of incipient movements. It may also explain the increased vulnerability to predation of mosquitofish, because they can be inhibited while trying to initiate a movement, or when a choice about how and where to run, or to whom and where to eat is to be made.

The decreased pattern of motor behaviors may also be related to low level of serotonin as pointed out by Smith and Weiss (1997). In an experiment dealing with predative behavior of fish (mummichongs), they found a significant increase of serotonin in the subject's brain. However, different from our findings, in their study the contamination of the subjects was done during the larval period. This "break" in the serotonergic system development occurs during the larval period, although as suggested by Tsai, Jang, and Wang (1995), cells of the serotonergic system may be mercurophilic, and therefore should tend to accumulate mercury and release it very slowly. Thus it seems plausible that mercury poisoning should decrease the levels of brain serotonin in mummichongs, consequently causing a reduction in predatory behavior. Zhou, Alder, Weiss, and Weise (1999) also indicated that the motor deficit may occur because thyroid function was modified by the Hg in the production of T4 hormone, as it has been reported by altering the motor function of fish (Godin, Dill, & Drury, 1974; Katz & Katz, 1978).

The work by Reed et al. (2006) helps to elucidate the physiological mechanisms of Hg action in the nervous system. According to the authors, mice infected during pregnancy with MeHg showed increased sensitivity to amphetamine (a dopamine agonist and noradrenergic), lower sensitivity to pentobarbital (muscarinic cholinergic receptors agonist) and clomipramine (serotonin agonist). The authors also indicate the lack of effect of haloperidol (dopamine reception blocker), suggesting that the effect is present in the neurotransmitter regulation levels, for example, in the generation, release and uptake, rather than acting on postsynaptic receptors. Reed et al. (2006) also indicated that the firing of midbrain dopaminergic neurons are strongly influenced by the presence of reinforcing consequences and suggest that these neurons may act in the elimination of choice options because of the presence of mesencephalic dopaminergic neurons. These mesencephalic dopaminergic neurons fire selectively when the reinforcement is greater than expected, whereas separate neurons fire when the reinforcement is less than expected. The above-mentioned effects of mercury, acting on the midbrain dopaminergic neurons, affects in turn the neurotransmitters regulation levels and seems to induce behavioral changes in situations that require choice.

Conclusion

In comparison with the control group, the toxic effect of $HgCl_2$ in water at a dose levelof 0.2 mg / L caused a decrease in motor activity and aggressive display of *Betta splendens* when they were exposed to the mirror test. Metallic mercury cause a reduction in movement emission in the progressive doses, however, the reduction became more pronounced in progressive rather than the acute dose condition. This motor activity decrease implies an impairment of feeding behavior, inhinbition of predators avoidance, reproductive behavior, mate choice and territoriality.

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