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A preliminary study about the effect of benzo[*a*]pyrene (BaP) injection on the thermal behavior and plasmatic parameters of Nile tilapia (*Oreochromis niloticus* L.) acclimated to different temperatures

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Abstract. Benzo[*a*]pyrene is a polycyclic aromatic hydrocarbon produced by naturally and anthropogenic sources and may be present in aquatic environments. Nile tilapias were acclimated to different temperatures (20, 25 and 30 °C), one group was injected with BaP (10 µg BaP g·fish^{-1}) and the other with the vehicle (DMSO) without contaminant. Thereafter, the thermal behavior and plasmatic parameters (cortisol, glucose and aspartate aminotransferase) of both groups were monitored. The thermal behavior of the tilapia was negatively affected by the injection of BaP. The critical thermal maximum (CTMax) of non-contaminated fishes were 40.1, 42.3 and 43.7 °C for those acclimated to 20, 25 and 30 °C respectively, whereas in BaP group the CTMax decreased (40.7, 41.3 and 42.6 °C). Plasmatic cortisol decreased in fish injected with BaP compared to those non-contaminated, also, the decrease was greater at higher temperatures. Glucose and aspartate aminotransferase increased as function of temperature, but the increases were higher in fish injected with BaP. The results suggest that BaP affects the thermal behavior, resistance and physiological status of Nile tilapia, but the effect increase as a function of temperature.

Keywords: Aquatic pollution, physiological status, polycyclic aromatic hydrocarbons

Resumen. Estudio preliminar del efecto de la inyección de Benzo[*a*]pireno en el comportamiento térmico y parámetros plasmáticos de la tilapia del Nilo (*Oreochromis niloticus* L.) aclimatada a diferentes temperaturas. Benzo[*a*]pireno (BaP) es un hidrocarburo aromático policíclico producido por medio de fuentes naturales y antropogénicas que puede estar presente en ambientes acuáticos. Tilapias del Nilo fueron aclimatadas a diferentes temperaturas (20, 25 y 30 °C), un grupo de ellas fue inyectado con BaP (10 µg BaP ·g· pez^{-1}) y otro con el vehículo (DMSO) sin contaminante. Después, se monitoreó el comportamiento térmico y parámetros plasmáticos (cortisol, glucosa y aspartato aminotransferasa) de ambos grupos. El comportamiento térmico de la tilapia fue afectado negativamente por la inyección de BaP. Las temperaturas críticas máximas (TCMax) de las tilapias no-contaminadas fueron 40.1, 42.3 and 43.7 °C para las aclimatadas a 20, 25 y 30 °C respectivamente, mientras que las tilapias inyectadas con BaP mostraron valores menores de TCMax (40.7, 41.3 y 42.6 °C). Los niveles de cortisol plasmático disminuyeron en los peces inyectados con BaP; además, la disminución fue mayor en las temperaturas más elevadas. Los niveles de glucosa y aspartato aminotransferasa se incrementaron en función del tiempo, pero el incremento fue mayor en aquellos peces inyectados con BaP. Los resultados indican que el BaP afecta en comportamiento y resistencia térmica, así como los parámetros plasmáticos de la tilapia del Nilo, aunque el efecto se incrementa en función de la temperatura.

Palabras clave: contaminación acuática, estado fisiológico, hidrocarburos aromáticos policíclicos

Introduction

The pollution and manipulation of the aquatic ecosystem are worldwide problems which increase over time and have demanded the attention of the scientific community (Lemly 2004; Denton *et al.* 2006; Boehm *et al.* 2007). Many ecosystems have been contaminated with pollutants from diverse sources. Several of those pollutants are toxic and can have negative effects on the physiology of fishes which inhabit those ecosystems. In addition, the toxic effects of chemicals can be influenced by various physicochemical factors, including temperature (Howe *et al.* 1994). Patra *et al.* (2007) affirmed that “the increase in production of toxic chemicals and the global warming phenomena become subjects of concern for ecologists in obtaining relevant knowledge on the tolerance of organisms to abiotic factors such as temperature”. Hence, it has been stated that when fishes undergo contaminated environments “the toxicity of particular chemicals increase as function of temperature” (Sprague 1985; Heath 1995).

Polycyclic aromatic hydrocarbons (PAH´s) are organic chemicals that bioaccumulate in the organisms, causing negative effects at cellular and molecular level (Arniç *et al.* 2000). Moreover, PAH´s are ubiquitous contaminants in environments that are frequently receiving mixtures of those chemicals from urban and industrial sources (Beyer *et al.* 1998; Ruddock *et al.* 2000).

Benzo[*a*]pyrene (BaP) is one of the most important PAH´s that have been studied and occurs through naturally and anthropogenic sources, such as forest fires and petroleum spills and its respective derivates (Hartl 2002). Its presence has been detected in surface water, tap water, rain water, ground water, waste water, and sewage sludge (U.S. EPA 1991). BaP is reported to affect aquatic animals and marine ecosystems (Hylland 2006) because their mutagenic and carcinogenic properties (Cerniglia 1984; Kanaly & Harayama 2000).

In nature, the fishes have the ability to thermoregulate, i.e. to choice optimal temperatures and discriminate sub-optimal ones (Armour 1991). Nevertheless the thermoregulation in fish may be modified by different environmental factors (e.g., season, photoperiod, age, light intensity, salinity, disease, nutrition, pollutants and biotic interactions) (Reynolds & Casterlin 1979; Kavaliers & Hawkins 1981; Baird & Krueger 2003; Ward *et al*. 2010). Furthermore the thermoregulation may be impaired by chemicals that affect the brain function, leading the fish to experience sub-optimal temperatures and increasing the risk of physiological damage or even death (Saadat *et al.* 2005).

Although the effect of some pollutants on fish thermoregulation has been documented, little or no information has been published about the effect of PAH´s on the thermal behavior, which would be an indicator of the effect of the pollutant on the physiology of different fish communities.

There are specific studies to observe thermal behavior of aquatic animals within a thermal gradient, where they choose optimal temperatures and discard sub-optimal ones (Hernández *et al.* 2002; Luna-Figueroa *et al.* 2003). Also, the thermal resistance may be a good indicator of the effect of any chemical on the fish physiology (Patra *et al.* 2007) and it is known as critical thermal maximum (CTMax), or “the temperature at which an organism loses the ability to escape from a condition that will lead it to death” (Paladino *et al.* 1980; Martínez-Porchas & Hernández-Rodríguez 2010). In addition, plasmatic parameters are frequently monitored in fish in order to evaluate their physiological and stress status.

The Nile tilapia (*Oreochromis niloticus* L.) is a species with a great culture potential in many tropical and subtropical regions all over the world (El-Sayed 1998). Also, it is widely used as a model in marine toxicology research because its resistance to stressful conditions (Atwood *et al.* 2001; Kosoff *et al*. 2009). The aim of this experiment was to determine the effect of BaP on the thermal behavior and resistance of the Nile tilapia acclimated to different temperatures.

Materials and methods

Organisms. The fishes were obtained from a commercial farm (Acuacultura del Desierto Farm) and transported into 25 L containers to the Centro de Investigaciones Científicas y de Educación Superior de Ensenada (CICESE). The organisms measured 19 ± 2.0 cm and weighted 155 ± 25 g.

Acclimation. Once in the laboratory, the fishes were randomly distributed into 18 tanks of 200 L (15 fish/tank; random design) and acclimated during 10 days to a constant temperature (25 °C), dissolved oxygen (DO): 5.55 mg·L^{-1}, pH: 7.8, total ammonia nitrogen (TAN): 0.25 mg·L^{-1}, photoperiod 12:12 and fed *ad libitum* with a commercial diet (Purina $^{®}$, 32 % protein).

The experimental tanks were divided into two groups, the non-contaminated group and the BaP group; each group was then sub-divided into three acclimation temperatures (20, 25 and 30 °C) and three tanks were assigned for every treatment or sub-division. To reach the experimental temperatures, the water in the tanks were cooled or heated at a rate of 1 °C·day^{-1} to achieve 20 and 30

°C respectively, while the tanks with water at 25 °C the temperature remained constant by using 250 watt heaters. When the experimental temperatures were reached (20, 25 and 30 °C), the organisms were acclimated during 15 days to those conditions. In the last day, the organisms from the contaminated group were intraperitoneally injected with BaP, while the organisms from the non-contaminated group were injected only with the innocuous vehicle Dimethyl Sulfoxide (DMSO, Sigma D-5879; David 1972) which is used also in cell cryopreservation (Kopeika *et al.* 2007).

Injection. Benzo(a)pyrene was dissolved in DMSO (0.5%) and intraperitonally injected (10 µg BaP ·g·fish^{-1}). For the non-contaminated organisms, only the DMSO without contaminant was injected. The concentration of BaP falls within the range of PAH´s that may be found in other fishes, preys and sediments from heavily contaminated sites (Huggett *et al.* 1988; Bender & Huggett 1987).

Thermoregulation. The thermal preference (TP) and resistance were measured twelve hours after the injection, considering that the ethoxy-resorufin-O-deethylase activity (EROD) and the concentration of total hydrocarbons in bile and brain were the highest according with preliminary tests (unpublished data).

To evaluate the thermal preference, two horizontal thermal gradients (length: 330cm, 15 virtual chambers) of acrylic were used. At one end of the gradient, water temperature was raised by two 1000 W heaters and at the opposite end cooled by a chiller (Cool-Flow HX-100) (Bückle *et al.* 2003); every virtual chamber had a thermal sensor that registered the temperature every 10 minutes. The thermal gradient was fixed at 18 - 33 °C.

Four tilapias of every tank were introduced into the gradients (4 fish/gradient) in the chamber that had a similar temperature than its previous acclimation. After 30 minutes, the observations began and the position of every fish and the temperature of those virtual chambers were recorded every 10 minutes during 2 hours. The thermal preference was established with base on the temperature of the chambers that the organisms visited more frequently. The same process was carried out for each one of the experimental units.

The thermal resistance was estimated by measuring critical thermal maximum (CTMax). Two fish of every tank were introduced into a 35 L aquarium with water that matched its previous acclimation temperature (2 repetitions/tank). After 30 minutes, the temperature was increased at a rate of 1°C·min^{-1} until the fishes showed onset spasms (OS). The temperature observed when the OS appeared was reported as the CTMax according to Lutterschmidt & Hutchinson (1997) criteria.

The acclimation response ratio (ARR) was calculated as the change in CTMax per Celsius degree change in acclimation temperature: ΔCTM/ ΔT (Claussen 1977).

Plasmatic parameters. Blood samples were also extracted 12 hours post injection. Prior to extraction, the organisms were anesthetized with 0.4 mL·L^{-1} of 2-phenoxyethanol for 5 minutes; samples were obtained by heart puncture technique (Shreck & Moyle 1990). Blood samples were placed in heparinized microtainer tubes and centrifuged at 5000 rpm during 10 min to obtain plasma. Thereafter the concentration of cortisol, glucose, and aspartate aminotransferase (AST) were measured in plasma by using commercial kits from Randox (Randox Laboratories, Oceanside CA., USA).

Management of experimental organisms. Once the thermal behavior and the OS were observed, the fishes were immediately transferred to aquariums with water at 30 or 35 °C and after 24 hours they were sacrificed with an overdose of anesthetic (2-Phenoxyethanol).

Statistical analyses. All data are presented as means ± standard deviations. One-way repeated measures ANOVA was performed to compare TP among non-contaminated and BaP treatments. For CTMax a simple One-Way ANOVA was carried out to compare BaP and non-contaminated groups. A two-way analysis of variance was carried out to evaluate plasmatic parameters. A significance level of α = 0.05 was considered and the software Statistica 6.0 was used to perform statistical analyses.

Results

Thermal behavior. No statistical differences were found among thermal preference of the non-contaminated and BaP groups (Table 1). However the thermal behavior of the organisms was influenced by the BaP injection and temperature. Fishes from non-contaminated groups showed a normal pattern of preferential behavior (i.e. they had a constant selection of temperatures trough time) in comparison with those injected with BaP, that did not discriminate any temperature. This was also observed in the variance of thermal preference data, since the variance in thermal selection of non-contaminated tilapias was 1.7, 0.7, and 3.0 °C, whereas in BaP ones was 6.4, 16.6 and 13.2 °C for the tilapia previously exposed to temperatures of 20, 25 and 30 °C respectively (Fig. 1).

The non-contaminated tilapia which were acclimated to 20, 25 and 30 °C chose temperatures within an interval of 3.4, 1.6 and 3.4 °C respectively when exposed to the thermal gradient, while the tilapia injected with BaP chose temperatures within an interval of 5.0, 8.6 and 7.8 °C respectively (Fig. 1), which could indicate a loss of orientation because the fish was not capable to locate an optimal interval of temperature.

The temperature of acclimation had a direct effect on the impairment intensity of thermal selection of organisms injected with BaP; tilapia acclimated at higher temperatures (25 and 30 °C) chose temperatures within intervals 72 and 56 % wider than those organisms acclimated to the lowest temperature (20 °C) (Table 1, Fig. 1) which means that higher temperatures enhance the loss of thermal orientation of tilapia.

Table I. Acute preferred temperature average of non-contaminated and BaP tilapia groups acclimated to different temperatures.

	Acute Thermal Preference (°C)
Non-contaminated 20 °C	28.6 ± 1.7
BaP 20 °C	28.3 ± 2.5
Non-contaminated 25 °C	30.6 ± 0.8
BaP 25 °C	28.5 ± 4.3
Non-contaminated 30 °C	29.4 ± 1.7
BaP 30 °C	28.3 ± 3.9

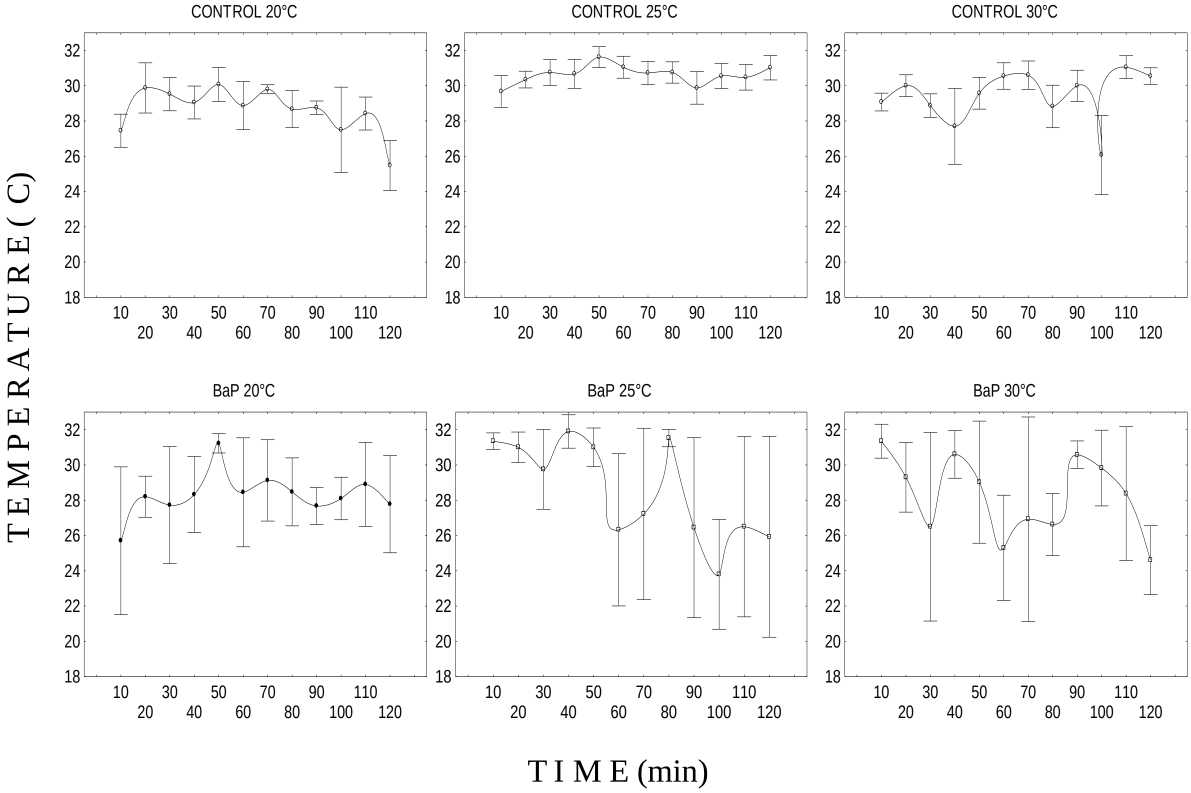


Figure 1. Acute preferred temperature ± standard deviation. Upper graphics show preferred temperatures of tilapias acclimated to 20, 25 and 30°C and injected with a vehicle (DMSO); lower graphics present preferred temperatures by tilapias injected with benzo[*a*]pyrene (BaP).

The thermal resistance was statistically influenced by temperature and BaP injection. A higher resistance was registered in organisms acclimated to elevated temperatures in both non-contaminated and BaP groups. With the exception of organisms acclimated at 20 °C, the CTMax decreased in organisms injected with BaP compared with those injected with a vehicle (Fig. 2). Moreover, the acclimation response ratio (ARR) in non-contaminated fishes was greater than that of fishes injected with BaP; ARR for non-contaminated tilapia was 0.43 and 0.36 *Δ°C CTMax/Δ°C* for the acclimation temperatures of 25 and 30 °C respectively, whereas the ARR in BaP injected organisms was 0.11 and 0.19 *Δ°C CTMax/Δ°C* respectively; thus, the acclimation capacity was diminished by the injection of BaP.

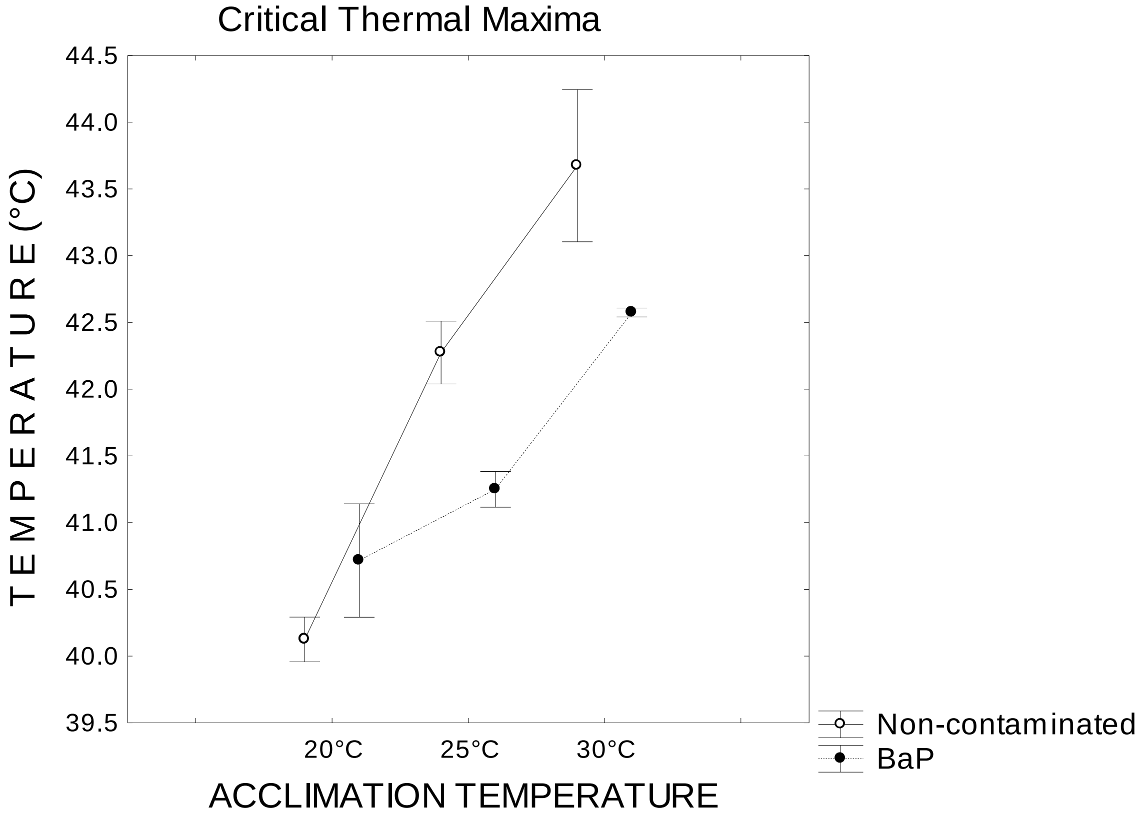
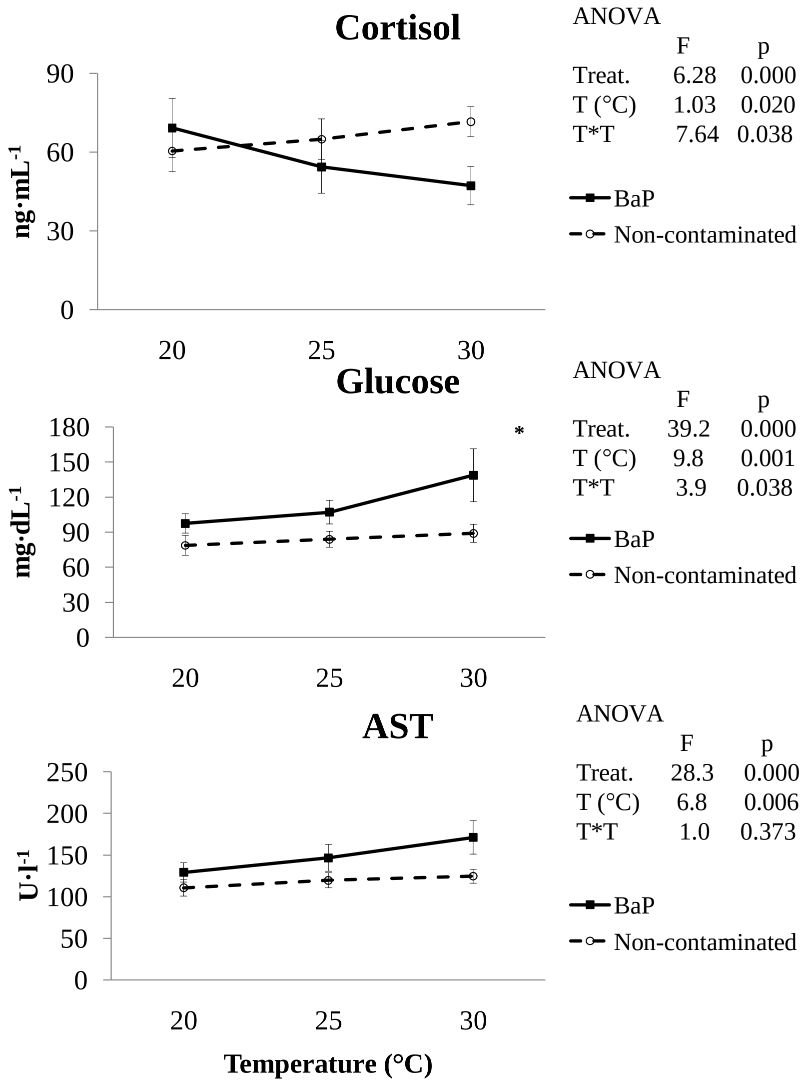


Figure 2. Temperatures where muscular spasms responses were observed in Nile tilapias injected with contaminant (BaP) versus vehicle (non-contaminated) acclimated at three different temperatures.

Hematological parameters. The hematological parameters were affected by either, the injection of the contaminant and/or the acclimation temperature.

The levels of cortisol were lower in BaP tilapia acclimated to the highest temperature (30 °C). In contrast, the cortisol levels in non-contaminated organisms increased as acclimation temperature did (Fig. 3). Glucose levels increased with temperature in all the treatments, but they were significantly higher in the contaminated organisms (BaP) compared with the non-contaminated ones for all acclimation temperatures. The glucose levels were 20, 8.6 and 24.8% higher in the BaP organisms acclimated to 20, 25 and 30 °C respectively, compared to those non-contaminated (Fig. 3). Regarding to AST, the levels increased in the contaminated organisms caused by both, the BaP injection and the acclimation temperature. No significant differences were observed at 20 °C and 25 ºC for among the BaP and non-contaminated groups, however the levels increased by 38% in the BaP group at 30 °C (Fig. 3).



ANOVA

	F	p
Treat.	6.28	0.000
T (°C)	1.03	0.020
T*T	7.64	0.038

ANOVA

	F	p
Treat.	39.2	0.000
T (°C)	9.8	0.001
T*T	3.9	0.038

ANOVA

	F	p
Treat.	28.3	0.000
T (°C)	6.8	0.006
T*T	1.0	0.373

Figure 3. Comparison of plasmatic parameters of Nile tilapia injected with benzo(a)pyrene (BaP), and injected with a DMSO vehicle (non-contaminated), and acclimated to different temperatures.

Discussion

Several authors have documented the presence of PHA´s in the habitat and culture systems of tilapia and other fishes (Hontela 1998; Roche *et al.* 2000; Kong *et al.* 2005; Liang *et al.* 2007). To this regard, Gold-Buchot *et al.* (2006) reported mass die offs of cultured tilapias in three lakes of Mexico due to the high pollution of organic chemicals (PHA´s).

In this experiment we found that the fish injected with BaP were not able to locate themselves within an optimal interval of temperature in contrast with the non-contaminated fish. The contaminated fish were disoriented, swimming from cold temperatures (22 °C) to hot ones (32 °C) without discrimination; apparently they were not able to differentiate between optimal (28 – 30 °C; Rakocy 1989) and suboptimal temperatures.

The presence of BaP has been documented in several tissues of teleost, such as: Gill, kidney, liver, gut, vasculature, pseudobranch, bile and brain, when the fishes have been exposed to contaminated waters or when they consumed food with BaP (Van veld *et al.* 1997; Ortiz-Delgado *et al.* 2009).

The brain is the principal organ that controls the thermal behavior of fishes; in fact, the neurons from the preoptic area increase their metabolic activity as a response to any thermal challenge (van den Burg *et al.* 2005). Deb *et al.* (2000) reported that PHA´s, specially fluoranthene and BaP, bioaccumulated mainly in brain and gonad of 11 fish species. In this experiment, it was observed twelve hours after injection, that the concentrations of BaP were the highest in brain and bile; therefore, it is possible that BaP affected the brain function of tilapia impairing the thermal behavior. According to that hypothesis, some authors have documented that PHA´s can cause neurotoxicity (Ritchie *et al.* 2001;

Tang *et al.* 2003) damaging the brain performance. Prosser & Nelson (1979) stated that fishes with damage in the preoptic region of the brain, had disruption on thermal behavior and regulation of internal body temperature, when they were introduced into a thermal gradient.

It was also observed that in BaP groups the higher acclimation temperatures (25 and 30 °C) increased the interval of preferred temperatures (e.g. disrupt thermoregulation) when compared to those acclimated to 20 °C; thus, BaP may be less toxic to fishes exposed to lower temperatures. Herein, some authors argued that fishes respond to cold temperatures or other severe environmental conditions by entering a state of dormancy (Walsh *et al.* 1983; Tiitu & Vornanen 2001) decreasing its metabolic rate and thus minimizing the damage by cold and the toxic effects of any pollutant (Rehwoldt *et al.* 1972). Also, it is well known that the toxicity of chemicals to aquatic organisms depends on the temperature (Cairns *et al.* 1975). In addition, the hypothesis of "higher damage at higher temperatures" was also supported by the response of plasmatic parameters.

Regarding to the thermal resistance (CTMax), it was influenced by the acclimation temperature; however this response has been widely documented (Currie *et al.* 1998; Li &Wang 2005; Martínez-Porchas & Hernández-Rodríguez 2010), because poikilotherms have the capacity to acclimate to cold or warm temperatures (Hochachka & Somero 1984; Huey & Bennett 1990). Del Rio-Zaragoza (2004) reported a CTMax of 40, 40.4 and 41.4 °C when acclimated *Oreochromis mossambicus* at 24, 28 and 32 °C respectively. However, another effect was evident during the resistance experiments, the CTMax was significantly ($P < 0.05$) decreased by the BaP injection, except in those organisms acclimated to 20 °C; furthermore the adaptation capacity (ARR) of fishes was also statistically diminished by the BaP injection. Some toxic chemicals may decrease the thermo tolerance and adaptation capacity of fishes (Carrier & Beitinger 1988; Patra *et al.* 2007), thus it is implied that fish populations living in environments contaminated with PHA´s might have less resistance to the increase of temperature.

Cortisol levels decreased in organisms injected with BaP. In contrast, increases of the hormone cortisol (or "stress hormone") have been observed in stressed fishes, because it activates glycogenolysis and gluconeogenesis processes in fish when the energy demand increases (Martínez-Porchas *et al*. 2009). However, it has been demonstrated that xenobiotics and other pollutants can be adrenotoxicants that impair cortisol secretion by interfering with the activities of steroidogenic enzymes (Hontela 1997). Apparently the impairment in cortisol secretion also occurred in tilapia injected with BaP and moreover, the impairment was greater at higher temperatures.

The levels of glucose, which is the principal energetic substrate, increased with temperature as expected; however the levels were significantly higher in the organisms injected with BaP; this might reflect an increase in the energetic demand, caused by a stressful situation. In addition, it has been reported increases in plasma glucose of fishes inhabiting xenobiotic polluted rivers (Palermo *et al*. 2008). Despite glucose increases in blood are commonly related with a raise in blood cortisol, such association was not observed in this experiment; however it is possible that other hormones may stimulate glucose release in the absence of cortisol (Martínez-Porchas *et al*. 2009).

The negative effect was also observed in the increase of AST, of the contaminated organisms. It is well known that increases of aminotransferase enzymes can be related to organ dysfunction or internal lesions in tissues; for instance, the damaged cells release their contents (including aminotransferases) toward the bloodstream and in consequence, the levels on these enzymes increase. The BaP increased the levels of AST at the highest temperature (30 °C), suggesting damage in any internal organ; in addition, lesions in liver, kidney and other organs have been detected in fishes exposed to PHA´s, in particular BaP has caused neoplasic lesions in these fish organs (Tuvikene 1995).

To date, there are no reports about the effect of PHA´s on the thermal behavior of fish; the results observed suggest that the BaP have a negative effect on the thermal behavior and physiological status of Nile tilapia. However this experiment only tested the effect of BaP intraperitoneally injected; it is necessary to carry our further studies in which the fish are exposed to contaminated waters and/or feed.

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