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Effects of subchronic postnatal exposure to chlorpyrifos (an organophosphate pesticide) on spatial learning

Efectos de la exposición subcrónica posnatal a clorpirifos (un pesticida organofosforado) en el aprendizaje espacial

Autor/a: Juan Antonio de las Heras Jiménez

Tutor/a: Luis Fernando Sánchez Santed

Abstract

Chlorpyrifos (CPF), a worlwide used organophosphate pesticide, an acetylcholinesterase inhibitor, has proved to be a disruptor of neurodevelopment, even a very low doses. There are several studies that found behavioural effects, like compulsivity or worsening in spatial learning. In this study, we proved that using a Morris Water Maze protocol, in which rats (as animal models) have to find a hidden platform, which protect them from water, in a water-filled pool using special cues. 2 groups of treatment (CPF-exposed or control) and 2 groups of sex (females and males) were made. CPF (or vehicle) was administered from PND10 to PND15, and rats were evaluated when were 14 months old. Results showed only a better performance in the Reinstating memory task of CPF- exposed rats, and a lower swim velocity. This could be due to a possible compensation mechanism that emerged in senectitude or to the proved interactions of CPF with steroid hormones metabolism, which may cause masculinization in females (and thus a better performance in some cases).

Key-words: Chlorpyrifos, acetylcholinesterase inhibition, spatial learning, senectitude, steroid hormones, masculinization, locomotor hypoactivity.

Resumen

El clorpirifos (CPF), un pesticida organofosforado usado mundialmente que inhibe la acetilcolinesterasa) ha probado ser un factor que altera el neurodesarrollo, incluso a dosis muy bajas. Hay estudios sobre efectos conductuales de la exposición a CPF, incluso a dosis muy bajas, como por ejemplo conducta compulsiva o déficit de aprendizaje espacial. En este estudio medimos esta capacidad mediante el laberinto de agua de Morris, en el que las ratas tienen que encontrar una plataforma oculta en una piscina de agua. Se formaron 2 grupos por tratamiento (CPF y control) y 2 por sexo (hembras y machos). El CPF (o vehículo) fue administrado desde el día posnatal 10 hasta el 15, y la evaluación se realizó cuando tenían 14 meses de edad. Los resultados mostraron solo un mejor rendimiento en una de las tareas por parte de las tratadas con CPF, y una menor velocidad de nado por éstas mismas. Esto podría ser debido a mecanismos de compensación que surgen en la senectud o a probadas interacciones con hormonas sexuales.

Palabras clave: Clorpirifos, inhibición de la acetilcolinesterasa, aprendizaje espacial, senectud, hormonas sexuales, masculinización, hipoactividad locomotora.

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1. INTRODUCTION

1.1. Organophosphates

Organophosphates (OPs) are considered one of the best-known and most used pesticide groups worldwide (Barberá, 1989). The first synthesis of a OPs was achieved in the XIX century, obtaining, in 1984, tetraethyl pyrophosphate by Philippe de Clermont (Marrs, in Karalliedde, Feldman & Henry, 2001). Since then, OPs were more extensively studied by the German chemical industry, finding out their powerful pesticide activity and even theorizing a possible use as neurochemical weapon. All OPs can be considered as phosphoric acid (H_3PO_4) products, and they can be classified by their chemical structure. Of course, their toxicokinetics will depend on those structures. Gupta (2006) reports "at least 13 types of OPs". Chemical structure of phosphoric acid can be seen in Figure 1:

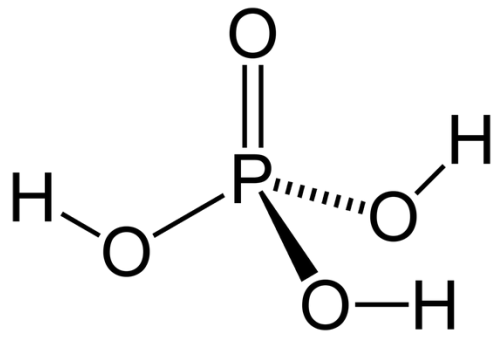

Figure: 1. Chemical structure of phosphoric acid (Retrieved from https://qph.ec.quoracdn.net/main-qimg-a5d7a8b12e416e684bed1d9ca737f06f)

Disel, Acikalin, Kekec & Sebe (2016) highlights the high liposolubility and good dermal and mucose absorption. The OPs mechanism of action consists in the irreversible inhibition of the cholinesterase (ChEs) enzymes, mainly the acetylcholinesterase (AChE) (butyrylcholinesterase (BuChE) too), which degrades by hydrolysis the acetylcholine (ACh; a neurotransmitter) in the synaptic cleft, giving choline and acetate as products (Carlsson, 2014). The ChEs activity inhibition produces an excessive accumulation of Ach in the synaptic cleft, originating over-activation in neurons with ACh receptors (muscarinic and nicotinic receptors). In that way there would exist, at least, 3 clinical categories derived from OPs intoxication (Yélamos, Laynez, & Pérez, 1999):

Acute cholinergic syndrome: Caused by the overactivation of cholinergic receptors (nicotinic and muscarinic), with symptoms like dyspnea, lacrimation, pulmonary edema, vomiting, diarrhea, incontinence, increased sweating, bradycardia, etc. There are more

symptoms, as cephalea, anxiety or ataxia, derived from affectation if other central nervous system (CNS) receptors.

Intermediate syndrome: Bleecker (in Karalliedde, Feldman, & Henry, 2001) describes it as a clinical profile with a beginning of 1 to 4 days after the cessation of cholinergic symptoms (even having been treated pharmacologically). Serious respiratory difficulties can be observed, caused by paralysis of cranial nerves controlled muscles.

Delayed neuropathy: It's considered a consequence of the "phosphorylation of an enzyme called neurotoxic esterase" (Yélamos, Laynez, & Pérez, 1999). Senanayake (in Karalliedde, Feldman, & Henry, 2001) describes it as a clinical profile that begins "about 1 to 3 weeks after acute exposure, and after a more uncertain interval following chronic exposure". Symptoms are mainly sensory-motor, affecting especially peripheral nerves, with an ascendant and retrograde evolution.

1.2. Chlorpyrifos

1.2.1. Chemical characteristics and toxicokinetics.

Chlorpyrifos (*clorpirifos* in Spanish; O,O-diethyl-O-[3,5,6,-trichloro-2-pyridyl] phosphorothioate in chemical nomenclature) or chlorpyrifos-ethyl is a chemical compound, xenobiotic, pertaining to the OPs group, currently used as pesticide (insecticide and acaricide) worldwide in big crops (Eaton et al., 2008), although there are reports of its utilization for biological control of vector-borne diseases (such as malaria, typhus or yellow fever) (Terry et al., 2003). It was introduced in market in 1965 (ATSDR, 1997) and it was used as fumigation agent in particular houses until 2002, when this use was completely restricted, given its probed negative consequences on neurodevelopment (Eaton et al., 2008). However, it still being extensively used worldwide (Casida & Quistad, 2004), being present in residual levels in a high percentage of foodstuff, due to its powerful pesticide activity in crops (Eaton et al., 2008). Chlorpyrifos (CPF), as said, belongs to the phosphorotiate sub-group of OPs. Generally, when we talk about CPF we are referring to the ethyl-form, existing an alternative form, which is less toxic and effective, but equally used and distributed in market, called chlorpyrifos-methyl. We show some of its physical-chemical characteristics in Appendix A, and we illustrate graphically the chemical structure of CPF in Figure 2, and Compound characteristics data are *mainly* taken from Barberá (1989), Eaton et al. (2008), Registro Estatal de Emisiones y Fuentes Contaminantes (n.d.) and Servicio de Prevención de Riesgos Laborales de la Universidad de Lleida (n.d.).

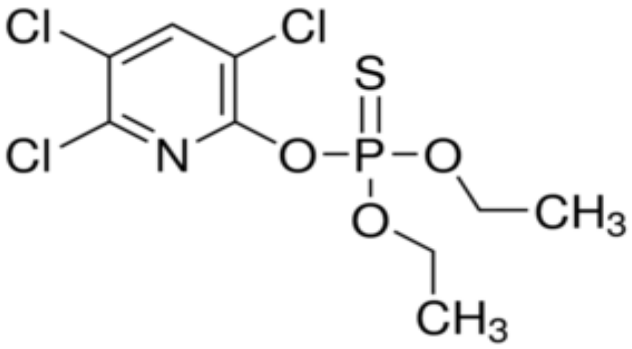

Figure 2. Chemical structure of CPF (Sigma-Aldrich).

In respect of the toxicokinetics of CPF (absorption, distribution, metabolism and excretion), it must be said that, obviously, factors such as age or species have an important influence. In that way, many studies have been carried out, in both humans and animals, and therefore they can give us some different data. Moreover, it must be said that the mainly used metabolite for measuring, especially, distribution, metabolism and excretion is 3,5,5-trichloro-2-pyridinol (TCPy) (however, diethylthiophosphate (DETP) or diethylphosphate (DEP) are commonly used too)). These metabolites are also utilized for measuring the absorption rate.

In relation to the **absorption**, it has been reported, in experimental animals, maximal levels of the previously mentioned metabolites "between 1 and 3 hours" after a CPF administration via oral (Timchalk, Busby, Campbell, Needham, & Barr, 2007; Timchalk et al., 2002). On the other hand, Nolan, Rick, Freshour, & Sanders (1984; as cited in Eaton et al., 2008) informed about "peak TCPy levels (…) after 6 h" in humans. There are data about dermal absorption (an important via of occupational exposure). Nolan et al. (1984; as cited in Eaton et al., 2008) reports "1.28 +/- 0.75% of the dermal applied dose of chlorpyrifos (…) recovered in the urine as metabolites after 24 h, as long as Meuling, Ravensberg, Roza, & van Hemmen (2005) talk about a TCPy peak in urine 72 h after the dermal application of 15 mg of CPF in humans.

About the **distribution**, Eaton et al. (2008) inform about a high lipophilic activity of CPF, and therefore a tendency to accumulate in adipose tissues (known in pharmacology as reservoir).

With respect of the metabolism of CPF, we can talk about two main metabolic routes in CPF biotransformation: the activation route and the inactivation route, existing authors that argue about implication of different organs in each one of these routes (Sultatos, Shao, & Murphy, 1983). However, the participation of the cytochrome P450 hemeproteins family is essential.

On the one hand, activation route consists on the transformation of CPF in an active metabolite (which is in fact the actual source of AChE inhibition): chlorpyrifos-oxon (Chambers, 1992; Smith et al., 2009). As said, this transformation is mediated and catalysed by cytochrome P450, and it's a reaction of oxidative desulfurization (Sultatos, 1994). Inactivation of this metabolite is mediated by the paraoxonase enzyme (PON), which originate, by hydrolysis, TCPy and DTP, which are inactive metabolites. Costa and Furlong (in Satoh & Gupta, 2010) described a PON1 activity that depends on its different isoforms. An important detail is that PON1 levels are especially low in new-borns, needing a period of time until it reaches desirable levels, in both humans (Cole et al., 2003) and rodents (Li, Matthews, Disteche, Costa & Furlong, 1997).

By the other hand, detoxication or inactivation route consists on the dearylation of CPF, also catalysed by cytochrome P450, generating DETP and TCPy as products (Jokanovic, 2001; as cited in Eaton et al., 2008). In the Figure 3 we graphically illustrate both metabolic routes (note that authors such as Eaton et al. (2008) don't consider in their drawings the intermediate metabolic):

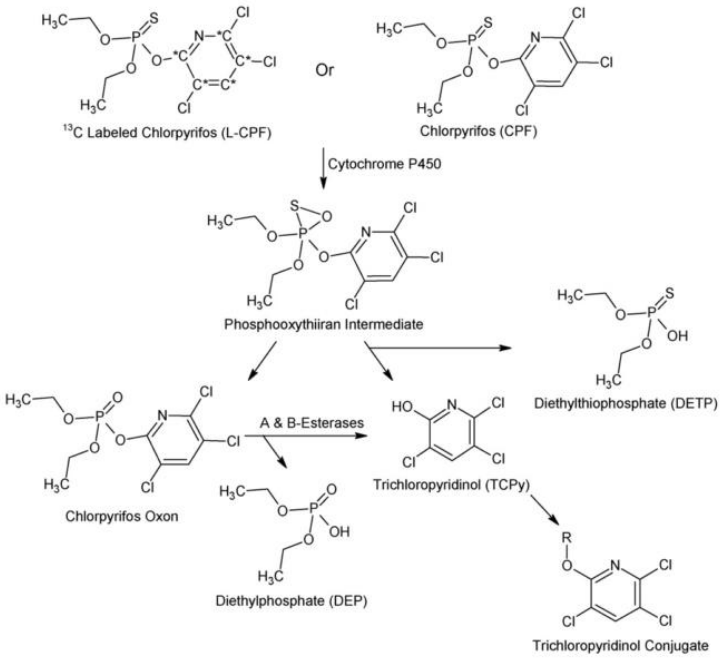

Figure 3. Activation and inactivation metabolic routes of CPF (Smith et al., 2009).

Finally, it's worth talking about CPF excretion. The main metabolite excreted by urine is TCPy (Smith, Watson and Fisher (1967) estimated a CPF excretion rate of 89% in TCPy. Bakke, Feil and Price (1976) also pointed out that "the glucuronide of 3,5,6-TCP was the principal urinary metabolite of chlorpyrifos in the rat" (as cited in Nolan et al., 1984). As said,

Nolan et al. (1984) also found, in voluntary human subjects, "peak TCPy levels (…) after 6 h". In addition, even though CPF excretion is essentially by urine, Hirom, Milburn, Smith and Williams (1972) talk about biliary or faecal excretion, too. Moreover, there are other studies that found out residues in maternal milk (such as Marty et al., 2007).

1.2.2. Mechanism of action of CPF and physiological effects

CPF, as the most part of OP compounds, has got an activity as irreversible inhibitor of cholinesterase enzymes, such as AChE or BuChE (this last with a big presence in plasma and liver). This inhibition has got as its immediate consequence the excessive accumulation of Ach in the synaptic cleft and a subsequent cholinergic over-activation. In this case, CPF has to be metabolized in CPF-oxon, the active CPF metabolite, in order to have any effect. CPF-oxon bonds its phosphoric radical to the esterase site of cholinesterase (ChE), in such a way that the enzyme is no more able to hydrolyse and degrade ACh (note that the chemical bond between ACh and AChE is in the anionic site).

Innumerable physiological effects have been described, commonly direct or indirect product of the inhibitory activity over the AChE, and amongst those are evident the typical parasympathetic-mimetic symptoms derived from the acute exposure to CPF; symptoms generally common to those consequent of intoxication by OPs with anti-cholinesterase activity. However, there are studies of the potential long-term effects of CPF exposure of pregnant women, mainly in very low doses via diet, in fetus neurodevelopment (i.e., in Rauh et al., 2006) (we should remember that CPF still being extensively used in crops as pesticide; see Eaton et al. 2008). In that way, it has been probed that CPF exposure could cause a long-term neurotransmission dysfunction in the cholinergic system, by down-regulation of muscarinic receptors (Pope, Chakrabortiu, Chapman & Farrar, 1992; Moser et al., 1998), although there are authors that find contradictory effects (Jett, Navoa, Beckles & McLemore, 2001).

Moreover, it has been established that CPF effects could go beyond AChE inhibition, suggesting possible dysfunctions in other neurochemical systems such as the serotonergic (Raines, Seidler, & Slotkin, 2001; Aldridge, Levin, Seidler & Slotkin, 2005; Aldridge, Meyer, Seidler, & Slotkin, 2005; Venerosi, Ricceri, Rungi, Sanghez & Calamandrei, 2010), GABAergic (Sánchez-Amate, Flores & Sánchez-Santed, 2001; Sánchez-Amate, Davila, Cañadas, Flores & Sánchez-Santed, 2002; Cardona, López-Grancha, López-Crespo, Nieto-Escámez, Sánchez-Santed & Flores, 2006), as can be deduced from Rastogi, Rastogi, Singhal & Lapierre (1985); dopaminergic (Aldridge, Meyer, Seidler, & Slotkin 2005; Slotkin & Seidler, 2007 (in doses insufficient to inhibit AChE activity, as cited in Venerosi et al.,

2010)), or even the endocannabinoid (Carr, Borazjany & Ross, 2011) one. In addition, there are descriptions of effects such as interferences in cellular signalling cascade (Huff, Corcoran, Anderson, & Abou-Donia (1994), as cited in Abou-Donia et al., 2003; Song et al., 1997) or, more globally, effects in the cellular division and development (Campbell, Seidler & Slotkin, 1997). Terry et al. (2007) found anomalies in neurotrophic factors and in axonal transport, whereas Slotkin, Brown & Seidler (2005) observed systemic effects such as hyperlypidemia and dysfunctions in insulin releasing. By other hand, López-Granero, Cañadas et al. (2013) and De Felice, Greco, Calamandrei & Minghetti (2016) have described oxidative stress effects of CPF exposure, and Peris-Sampedro et al. (2015) revealed interactions with human apolipoprotein E (apoE) polymorphisms.

1.2.3. Behavioural effects of CPF exposure

With regard to the behavioural consequences of CPF exposure (with an special emphasis in neurodevelopment stages), there are proofs of a possible anxiolytic effect of CPF (Aldridge, Levin, Seidler, & Slotkin, 2005; Carr et al., 2011), yet there also are studies with opposite results (Sánchez-Amate et al., 2001). It also has been proved that CPF promotes compulsive behaviour (Cardona et al., 2006; Montes de Oca et al., 2013). It has been observed alterations by locomotor hyperactivity too (Grabovska & Salyha, 2015; Levin et al., 2002; Yan, Jiao, Zhao, Yang & Peng, 2012), although other researchers have given contradictory data (Carr et al., 2001), surely due to differences in administration and behavioural evaluation methodology. Additionally, authors such as Grabovska and Salyha (2015) or Middlemore-Risher, Buccafusco and Terry (2010) found prototypical ADHD (Attention Deficit Hyperactivity Disorder) in rodents exposed to CPF. Moreover, Chanda and Pope (1996) have demonstrated changes in instinctive and reflexive behaviour. There are proofs of alterations in socio-emotional behaviour, such as those provided by Venerosi et al. (2010).

Deleterious effects such as those previously mentioned, in rodents, have been replicated in cohort studies, mainly summed up by Eaton et al. (2008) or Li, Lowe, McIntosh & Mink (2012), who considered that enough evidence has been found think that it can be generalized between species. For example, Rauh et al (2006) have reported an increased prevalence of mental/motor retardation and ADHD amongst children exposed to CPF. However, these last studies are epidemiological, and need experimental evidence to be verified. This evidence is provided, mainly, by experimental animal studies, such as those already discussed.

In respect of the consequences of CPF exposure on spatial learning and spatial memory, it must be said that many studies have been carried out, the most part using as paradigm or experimental protocol the Morris Water Maze (MWM), basically consisting on a water-filled

round pool, in which the animal has to find a platform, commonly invisible, that protect the animal from the water (it's based on the premise that the animal, rodent, has got preference by dry rather than wet environment). However, there also are experiments using other protocols, like the Radial Arm Maze (ARM), which measures reference and working spatial memory (Johnson, Chambers, Nail, Givaruangsawat & Carr, 2009) through the entries of animal in different arms (some of them baited) that begin in a central point, in a manner that enter twice or more times in the same arm is considered as working memory error, while enter in an arm that has never been baited is considered as working memory error.

In the Appendix B we show the most relevant articles about CPF-exposure and spatial learning evaluated with MWM in rodents, while in Appendix C we show data about CPF-exposure and spatial learning evaluation with ARM.

About methodology of these studies, it must be noted if exposure to CPF was carried out during the neurodevelopment of the subject, given that in this case the different neurochemical systems are specially sensible and vulnerable to environmental agents. In many cases, authors try to demonstrate, in addition to the spatial learning deficit, concomitant damage in hippocampal areas, such as in Terry et al. (2003), Gómez-Giménez et al. (2017) or Mullins et al. (2013), which is postulated as cause of the spatial learning dysfunction. Locomotor activity is also commonly evaluated in this studies, such as in Yan et al. (2012). However, it must be said that available data are contradictory, although this contradictory data could be reflect of different administration, dosage and behavioural evaluation methodologies. In that way, Terry et al. (2003) found a worse spatial learning with low doses, in the same direction of another study organised by the author (Terry, Beck, Warner, Vandenhuerk, & Callahan, 2012). Gómez-Giménez et al. (2017) also described spatial learning alterations after a prolonged prenatal CPF exposure. Also in Spain, Sánchez-Santed, Cañadas, Flores, López-Grancha, & Cardona (2004) observed a worse performance in MWM task after 2 sub-acute CPF expositions. A year later, Cañadas, Cardona, Dávila, & Sánchez-Santed (2005) only found an unstable learning curve in CPF-treated rats in comparison to control rats. There are some articles amongst these studies in which AChE wasn't significantly inhibited (such as in Yan et al., 2012), with the consequent causal implications.

We also have to talk about researches organised by López-Granero. In 2013, López-Granero, Cañadas, et al. found a worse performance in spatial learning of rats treated with a single dose of CPF, in the Reversal task of MWM (which basically measures cognitive flexibility), a short time after exposure. However, the same year López-Granero, Cardona, Giménez, Lozano,

Barril, Sánchez-Santed et al. didn't observe this deleterious effect when rats were exposed with low doses in a prolonged time. In 2014, López-Granero, Cardona, Giménez, Lozano, Barril, Aschner et al. treated rats with a sub-acute dose, finding almost no differences 72 after exposure, yet they did find it 23 weeks later. At last, López-Granero, Ruiz-Muñoz et al. (2016) didn't find evidence of any worsening in spatial learning after a prolonged exposure period, with low doses and a 7 months washout.

Moreover, Jett et al. (2001) described a worse spatial learning performance in weaned rats exposed to CPF, which didn't show any significant AChE inhibition. Mullins et al. (2015) also registered dysfunction in spatial learning performance in a MWM task, in rats prenatally CPF-exposed (though we have to note that they used a breed which haven't got the same neurodevelopment periods as Wistar rats, mainly used in studies described in the two paragraphs above), additionally presenting structural brain anomalies. Furthermore, Mamczarz et al. (2016) also observed spatial learning deficit after prenatal CPF-exposure.

However, as long as we know, there are no studies limiting CPF administration, in low doses (NOAEL, "no-observable-adverse-effect levels; see Eaton et al., 2008), to the short postnatal period in which processes of synaptogenesis are developed; as well as evaluating spatial learning (with a MWM protocol) when rats are in late adulthood (almost senectitude). Our hypothesis is that early postnatal exposure, in synaptogenesis stage, to CPF levels not enough to inhibit ChEs in a significant way (see, for example, Yan et al., 2013) will have, as consequence, a worse spatial learning and memory performance than control rats, all measured through a MWM protocol in the late adulthood age.

2. MATERIALS AND METHOD

2.1. Animals

20 Time-pregnant females Wistar rats arrived to our facilities at 5 days to parturition. They were fed ad libitum and water was disposed as well for free access. 19 out of the 20 rats gave birth. A total of 190 neonates (50% females) were pseudo-randomly distributed to their definitive dam, with a total of 10 neonates (50% females) for each mother. Neonates were sexed on PND1 (the day of redistribution), PND4, PND9 (one day before exposure protocol started) and, finally, at PND21 (weaning day). On PND21, young animals were weaned and pseudo-randomized redistributed (rats at the same home-cage must be of the same sex, from different original mother and from different assigned Dam) 4 per home-cage, in a temperature

and humidity controlled room (22 +/- 2 º C; 50 +/- 10%) and a light/dark cycle of 12 hours (13:30h/01:30 h). For this experimental protocol, 32 animals (50% females) were chosen.

Experiment began when rats were on PND 10 in respect of treatment administration, whereas spatial learning evaluation protocol began when rats were 14 months old. From PND 70, they received a restricted food diet (13 g per male and 11 g per female), and water *ad libitum*. Food per animal was increased, from the beginning of spatial training tasks, to 14g per male and 12g per female. Rats weighted a mean of 356,68g (σ = 72,95) the immediately previous week to the beginning of spatial training evaluation (by sex, females weighted a mean of 297,27g (σ = 10,01) and males a mean of 412,38g (σ = 12,25)). Procedures were approved by the animal bioethics committee of the University of Almería, and they were adjusted to the Spanish Royal Decree 53/2013 of 1 February and to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on experimental animal protection and comfort.

2.2. CPF administration and dosage

CPF was purchased as PESTANAL®, from Sigma-Aldrich (ref. no. 45395; purity > 99%). Animals were divided into 2 different groups depending on the treatment (CPF group or control group), and into 2 groups depending on the sex (female or male). Thus, there were 4 sub-groups (CPF-female, CPF-male, control-female and control-male), each one with 8 rats. CPF in experimental condition was administered via gavage in a dose of 1 mg/kg (not enough to cause significant ChEs inhibition; see Yan et al. (2003)), dilute in corn oil, whereas rats in control condition only received corn oil (vehicle) via gavage, too. Both CPF and vehicle were administered daily from PND 10 to PND 15.

2.3. Apparatus

Spatial learning and memory were measured in a round black pool, height of 50 cm and diameter or 150 cm, filled with clear water, and maintained in a constant temperature between 22 and 23 º C. There were 12 holes in the pool base, which allowed to place a black escape platform (height of 38,5 cm and diameter of 10 cm). The pool was divided into 4 quadrants (A, B, C and D), as the following figure (Figure 4) shows:

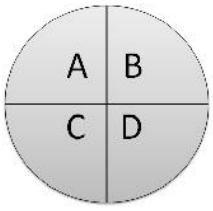

Figure 4: Disposition of quadrants in MWM pool.

2.4. Procedure in MWM

2.4.1. Spatial training task (session 1 - 8)

We have based the spatial learning evaluation methodology on that used by de Bruin, Sánchez-Santed, Heinsbroek, Donker & Postmes (1994). We placed a submerged (2,5 cm below the water) platform in one of two possible positions, quadrant B or C, in a manner that remains invisible for the rat, which have to reach the platform. Each animal was assigned to a platform position (B or C), which remained constant in all trials and sessions. The animal was released from one of the four quadrants, which remained the same in all animals by each session, changing pseudo-randomly between sessions, in a manner that each quadrant was used twice in this first phase. The animal had got 90 seconds to reach the platform, and it was allowed to remain in there for 30 seconds, completing the trial after that. In case that the rat hadn't reached, the animal was taken and placed over the platform for 30 seconds too (it scored 90 seconds). Each session consisted on 4 trials per animal. Once the 4 trials were completed, the rat was dried with a towel and it was put in the cage. One session was carried out per day. Escape latency (s), distance moved (cm) and swim velocity (cm/s) were measured.

2.4.2. Transfer test (session 9)

Platform was removed and rats were allowed to swim freely for 30 seconds. Release point was the opposite quadrant to its platform in the Spatial training task (e.g., if rat had had its platform in B, its release point in Transfer was quadrant C). Time spent in each quadrant (s), distance moved (cm) and swim velocity (cm/s) were measured.

2.4.3. Reinstating memory (session 10)

Platform was again placed (submerged) in its original position for each animal, in order to carry out for each rat a block of 4 trials, identical to the Spatial learning task. Release point was C for all animals in all trials. Time procedure was the same as that one followed in the first phase (90 seconds – 30 seconds). Escape latency (s), distance moved (cm) and swim velocity (cm/s) were measured.

2.4.4. Reversal training (session 11 – 13)

Position of training platform of each animal was inverted, in a way that those who had had assigned platform in B changed its target to C (opposite quadrant). Release point varied between sessions, but it remained the same for all animals in the 4 trials. 4 trials by session per animal were carried out, one session per day. We followed the same time procedure of Spatial training task, and we measured the same variables. This task is aimed to measure cognitive flexibility.

2.4.5. Visually-cued task (session 14 - 16)

In this task, the platform wasn't invisible, but remained 1,5 cm over the water (in addition, it was grey coloured), in a way that it was visible to the rat. Platform position changed between trials, but the sequence of change (e.g. A → B → C →D, in session 14) remained the same for all animals in each session. This sequence changed between sessions. Release point was the opposite quadrant to that which contained the platform (by each animal) in the Spatial training task. There was one session per day. We followed the same time procedure of Spatial training task, and we measured the same variables, too.

2.5. Measurements of ChEs activity

We based our ChEs measurement procedures on Moreno et al. (2008). Firstly, we homogenized pellets, at a ratio of 1/10 (w/v), with Triton X-100 (1%), in 0.1 M sodium phosphate buffer (pH = 8). Then we centrifuged the homogenate for 15 minutes at 15,000 x g. We threw the pellet out, and we used the supernatant to carry out the ChEs assay, of which activity was measured using a modification of the Ellman method (Ellman, Courtney, Andres, & Featherstone, 1961), utilising a 96-well microplate reader (DTX 880, Beckman Coulter). We diluted supernatant with 0.1 M sodium phosphate (pH = 8) (ratio 1/10 (v/v). After that, we mixed 10μL of this dilution with 221 μL of sodium phosphate buffer (0.1M, pH = 8) and 60 μL(in 0.1M sodium phosphate buffer; pH = 8) of 5, 5-dithiobis-2-nitrobenzoic acid (final concentration = 0.33mM). We carried out an incubation for 300s (at 37ºC), and then we added 9 μL of acetylcholine iodide (pH = 8, dilution in 0.1 M sodium phosphate buffer, final concentration = 0.5mM). Then, we monitored the reaction rate (at 37ºC) for 22 minutes, and measured absorbance at 30 seconds intervals, at 405 nm (3 seconds shake before reading, 45 cycles). When we analysed slopes, we selected 2 cycles (60 seconds). We calculated the enzymatic activity as the rise in absorbance using the equation of Ellman et al. (1961). Finally, we measured protein concentration as Bradford (1976).

2.6. Statistical analysis

We performed 3 analyses of variance with repeated measures (rmANOVA) in Spatial training (+ Reinstating memory), Reversal training y Visually-cued task, with the within-subject variable SESSION, and SEX and TREATMENT as between-subject factors. In Transfer task, we carried out a multivariate analysis of variance (ANOVA), with POSITION (B or C training platform) as co-variable, and SEX and TREATMENT as between-subject factors. We used the Sidak correction for multiple comparisons and post-hoc analyses in ANOVA. The accepted level of significance for analyses was $p \leq 0,05$. All analyses were performed with SPSS Statistics 22 (IBM).

3. RESULTS

3.1. Cholinesterases (ChEs) measurement

As it can be observed in Figure 5, little inhibition of ChEs on PFC was noticed in CPF exposed animals from non-exposed ones. In this way, female rats showed the largest inhibition (around 12%), meanwhile males did not reach 7%. Only a soft tendency was observed for TREATMENT condition [F (1, 16) = 3.473, p = 0.081], but nothing in TREATMENT X SEX interaction [F (1, 16) = 0.319, p = 0.580]. Otherwise, different evolution was observed by SEX 6 days after exposure to the xenobiotic had ended, with a clear maintenance on ChEs from controls in male rats (6% less compared to control rats), but an important "rebound" effect on females (increase of 14% respecting control rats). In this way, not even a tendency was observed for TREATMENT [F(1,16)= 0.439, p= 0.517]o such TREATMENT X SEX interaction [F(1, 16) = 2.668, p = 0.122]. No significant data was obtained with rmANOVA linking both time criteria, but this information is not presented here because it was considered by the authors as no appropiate (both days were analysed on different plates, thus different Spectrophotometer running).

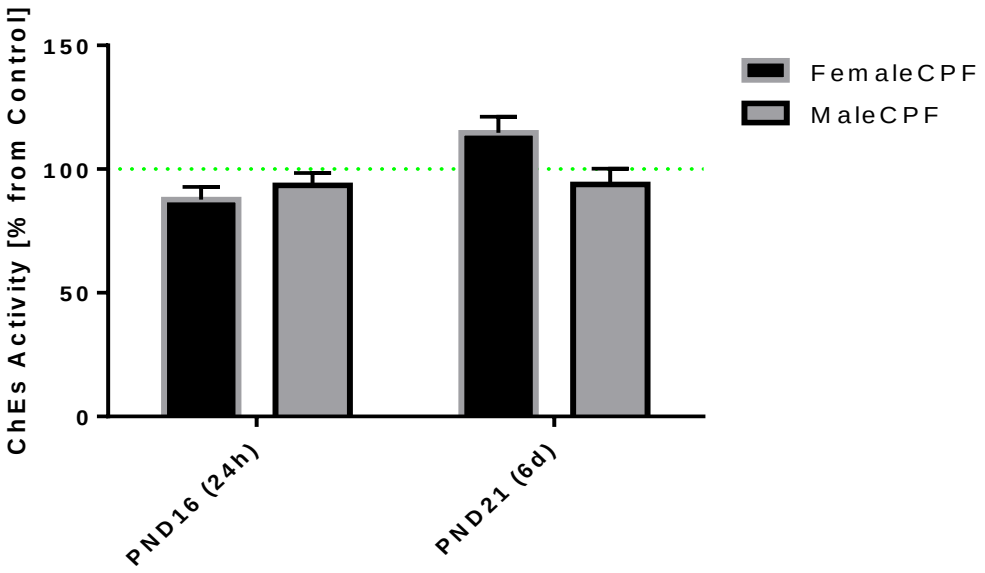

Figure 5. ChEs activity (% from control) on PND 16 (24h) and PND 21 (6d) by sex.

3.2. Morris Water Maze

3.2.1. Spatial training task

Escape latency (s) was analysed with SESSION as within-subject factor, and SEX and TREATMENT as between-subject factors, for CPF-treated group (CPF FEMALES and CPF MALES) and control group (CORN FEMALES and CORN MALES). Figure 6 shows the

learning curve for all groups. There was significant effect of SESSION [F (7, 189) = 49.788; p < 0.001], meaning that rats learnt and performed better the task across sessions. There weren't significant interactions between SESSION and between-subject variables (SESSION X SEX [F (7, 189) =1.744, p = 0.101], SESSION X TREATMENT [F (7, 198) = 0.687, p = 0.683, SESSION X SEX X TREATMENT [F (1, 27) =1.077, p = 0.309]). Moreover, neither SEX [F (7, 198) = 0.488, p = 0.842] nor TREATMENT [F (1, 27) = 1.512, p = 0.229] had significant effects in escape latency. There wasn't significant effect of SEX X TREATMENT interaction [F (1, 27) =1.077, p = 0.309], either.

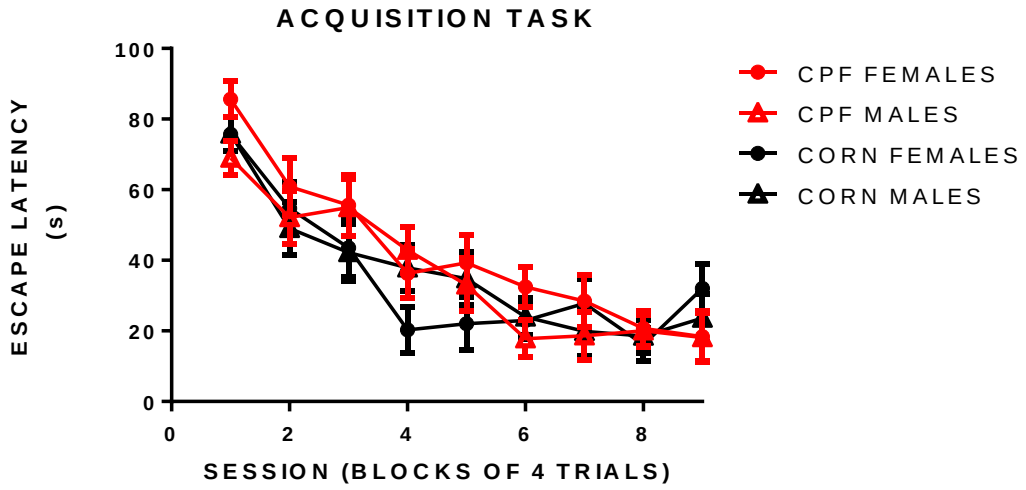

Figure 6: Mean escape latencies (s) of each sub-group in the Spatial training phase (session 1 to session 8) and *reinstating memory* task (session 9).

We also compared, in the Spatial training phase, the 1st and 8th sessions (fig. 7). We only found a significant effect of SESSION [F (1, 27) = 210,704, p < 0.01]: rats performed in the 8th trial better than in the 1st one.. Interactions of this variable with SEX [F (1, 27) = 0.122, p = 0.730] and TREATMENT [F (1, 27) = 1, 769, p = 0.195] didn't have any significance. We analysed another within-subject variable, TRIAL, but it didn't show significant effects [F (3, 81) = 1,079 p = 0.363]. No significant SESSION X TRIAL interaction effect was found [F (3, 81) = 0,252, p = 0.860]. On the other hand, we found significant effects of SEX [F (1, 27) = 5,126, p = 0.032] and SEX X TREATMENT [F (1, 27) = 8,545, p = 0.007], but, since this data of escape latencies consisted on the mean of the two sessions, there is no possible statistical justification to interpret that.

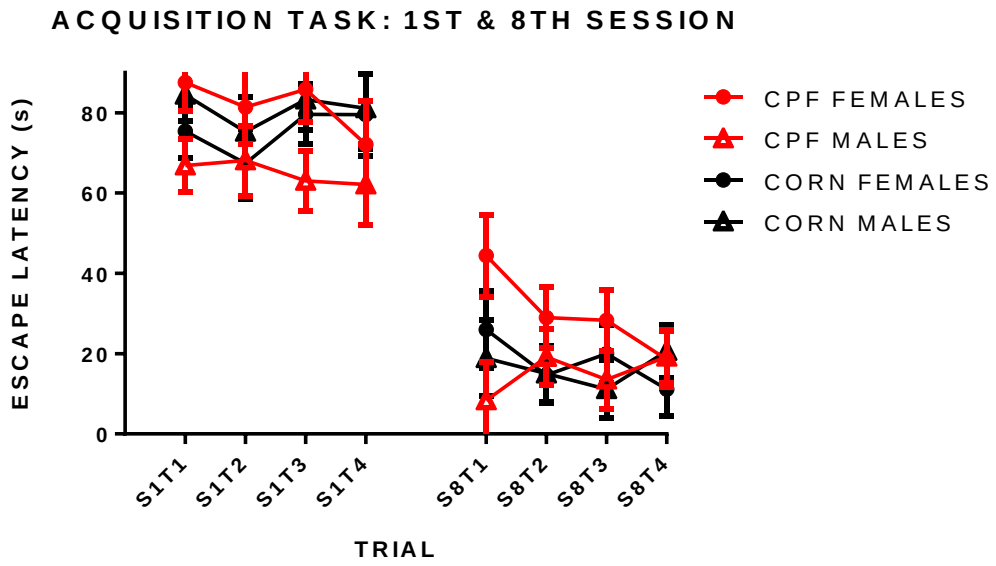

Figure 7: Mean escape latency (s) comparative of session 1 (S1T1 – S1T4) and session 8 (S8T1 – S8T4) (Spatial training phase).

3.2.2. Transfer task

Multivariate ANOVA analysis was performed for the Transfer test. None significant effect was found, neither for TREATMENT [F (4, 24) = 0,496, p = 0.738] or SEX [F (4, 24) = 0,642, p = 0.638], nor for the interaction TREATMENT X SEX [F (4, 24) = 0,549, p = 0.702]. We illustrate the obtained data in Figure 8:

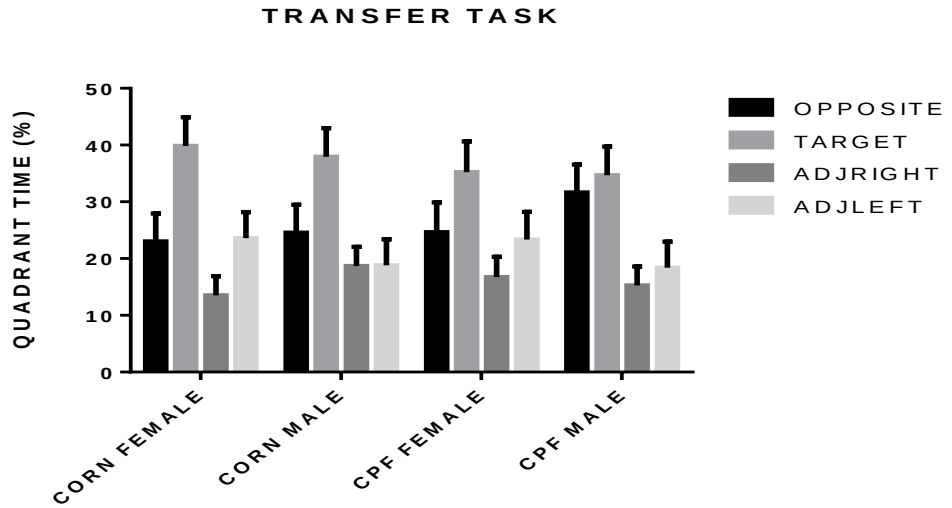

Figure 8: Percent of time spent in each quadrant for each of the 4 sub-groups. We codify the platform trained in Spatial training phase as TARGET.

3.2.3. Reinstating memory task

In order to evaluate the possible negative effect of the Transfer test (remind that, in that phase, platform was removed), we analysed the last session of the Spatial training phase in comparison to the Reinstating memory task. We can see the overall results in the Figure 9:

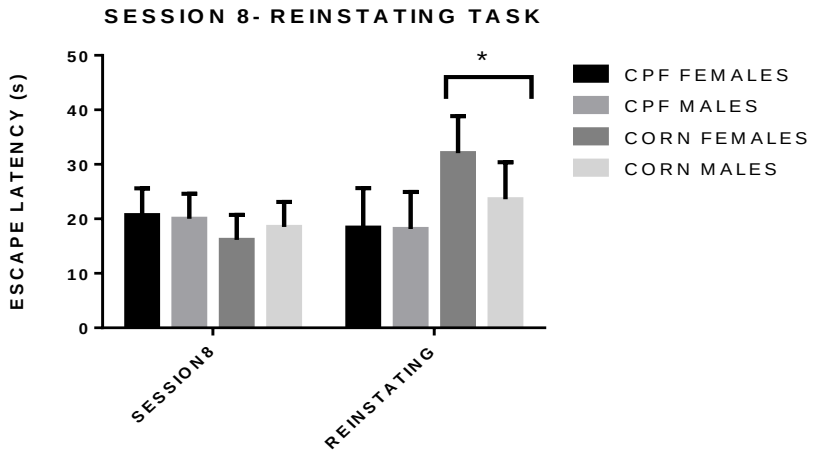

Figure 9: Mean escape latencies (s) in the last session of Spatial training task and in the Reinstating task.

Overall, there was no significant effect of the within-subject variable S8REINST [F (1, 27) = 1.955, p = 0.173]. In addition, we didn't find significant effect of S8REINST X SEX [F (1, 27) = 0,745, p = 0.496] or S8REINST X TREATMENT X SEX [F (1, 27) = 0,858, p = 0.362]. However, S8REINST X TREATMENT interaction was significant [F (1, 27) = 4,342, p = 0.047]. Post-hoc analyses revealed that rats in control group had got a significantly worse performance in the Reinstating memory task when compared to the performance of the last session of Spatial training task, hence being negatively affected by the Transfer task. In addition, although there wasn't a significant effect of S8REINST X TREATMEN X SEX, we observed that females group had practically the total weight of the lower control group performance. Therefore, in females only the CPF treated group did not show a worsening of performance after the Transfer task. . In order to know if there was any influence of the trial (recall that each session had got 4 trials), we performed a second analysis with the within-subject variable TRIAL, but we didn't find any significant effects of this variable [F (3, 81) = 1,946, p = 0.129]. Effects of interaction weren't significant, either (TRIAL X SEX: [F (3, 81) = 0,246, p = 0.864], TRIAL X TREATMENT: [F (3, 81) = 1,235, p = 0.303], TRIAL X SEX X TREATMENT: [F (3, 81) = 0,558, p = 0.645], TRIAL X SESSION: [F (2.264, 61.115) = 0,750, p = 0.492], TRIAL X SESSION X SEX: [F (2.264, 61.115) = 1,857, p = 0.160], TRIAL X SESSION X TREATMENT: [F (2.264, 61.115) = 0,713, p = 0.510], TRIAL X SESSION X SEX X TREATMENT: [F (2.264, 61.115) = 0,275, p = 0.787]. In respect of the

between-subject variables, none of them showed significant effect (TREATMENT: [F (1, 27) = 0,416, p = 0.524], SEX: [F (1, 27) = 0,119, p = 0.732], TREATMENT X SEX: [F (1, 27) = 0,065, p = 0.800]).

3.2.4. Reversal task

We analysed the data including the within-subject variable TRIAL. Rats had a general improvement in its performance across the sessions, as we can see in Figure 10, as SESSION effect was significant [F (2, 54) = 8,223, p = 0.001]. TRIAL effect was significant, too [F (3, 81) = 15,419, p < 0.001]. However, there was neither significant effect of SESSION X TRIAL interaction ([F (3.998, 107.954) = 1,137, p = 0.343) nor significant effects of SESSION X between-subject variables (SESSION X SEX: [F (2, 54) = 0,499 p = 0.610], SESSION X TREATMENT: [F (2, 54) = 0,021, p = 0.979], SESSION X SEX X TREATMENT: [F (2, 54) =0,947, p = 0.394]. In respect of the between-subject variables, there weren't significant effects, either (SEX: [F (1, 27) = 0,430, p = 0.517], TREATMENT: [F (1, 27) = 0,003 p = 0.958], SEX X TREATMENT: [F (1, 27) = 0,106, p = 0.747].

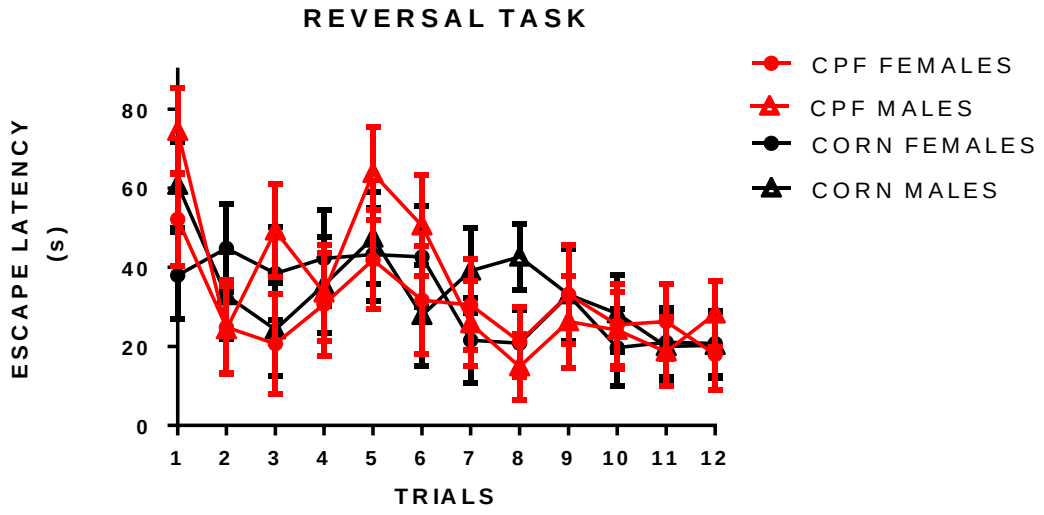

Figure 10: Mean escape latency (s) by trial and session (S1: T1-4, S2: T5-T8, S3: T9-T12) for each sub-group in Reversal task

3.2.5. Visually-cued task

Finally, we show the results (as can be seen in Figure 11) obtained in Visually-cued task. On the one hand, there was a general improvement of rats performance between the sessions (SESSION: [F (2, 54) = 5,135, p = 0.009]). On the other hand, there wasn't significant effect of any interaction of SESSSION and between-subject variables (SESSION X TREATMENT: [F (2, 54) = 0,827, p = 0.423], SESSION X SEX: [F (2, 54) = 0,092, p = 0.912], SESSION X TREATMENT X SEX: [F (2, 54) = 0,316, p = 0.730]). Furthermore, none of between-subject

variables showed significant effect (TREATMENT: F (1, 27) = 0,764, p = 0.390], SEX: F (1, 27) = 0,090, p = 0.766], TREATMENT X SEX: F (1, 27) = 2,844, p = 0.103]).

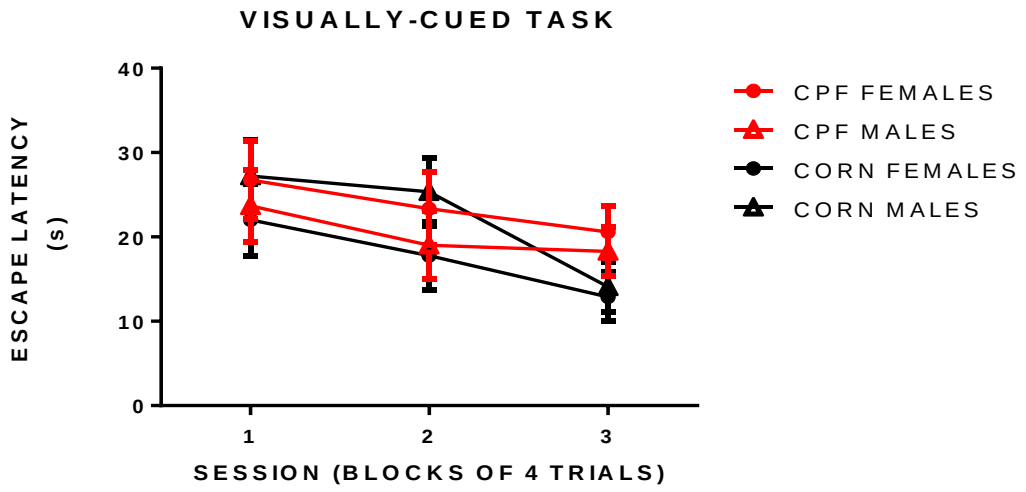

Figure 11: Mean escape latency (s) by session for each sub-group in Visually-cued task.

3.2.6. Control variables

In addition, total distance moved by the rats (cm) and mean velocity (cm/s) in each trial by session were analysed. We show the data in Appendix D, and graphics in Appendix E. Neither treatment nor sex had got significant effect on total distance moved or mean velocity ($p > 0.05$) in almost all sessions. However, we found a significant effect of TREATMENT on velocity in the Visually-cued task [F (1, 27) = 5,562, P = 0.056]: the CPF-treated group showed a lower velocity compared to the control group. Data can be graphically seen in Figure 12:

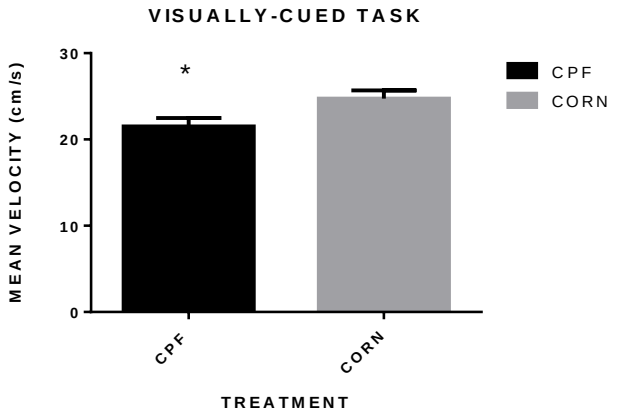

Figure 12: Mean velocity (cm/s) by each treatment group in the Visually-cued task.

As we can see in Appendix E, although there are no significant treatment effects in other task, a trend towards lower mean velocity in CPF-exposed compared to that of the control group can be observed.

4. DISCUSSION

Our hypothesis was only partially confirmed in the case of the comparison between performance in Reinstating memory task and the last session of the Spatial learning task. Thus, we found that, after completing the Transfer task, control rats had a worsening in their spatial learning performance, whereas CPF-treated rats performed in a normal way. Moreover, although we can't talk about significant differences between sexes in these control rats, we found that almost all the weight of the worsening effect may be attributed to the female rats, as they had higher escape latencies than males. Maybe we could have found significant differences increasing the number of rats by sub-group. In addition, when we analysed control variables, a general decrease in velocity was found in CPF-treated rats, in comparison to control rats, in one of the performed task (additionally, it seemed that there was a trend towards a velocity decrease in rats exposed to CPF). Moreover, the dose of CPF administered almost didn't inhibit ChE activity, so we can't attribute the found effects directly to a dysfunction in the ACh metabolism.

We are going to talk firstly about the locomotor effect. As said, we observed a velocity decrease in CPF exposed rats, something that could be possibly interpreted as an anxiolytic effect. We have found other studies which provide evidence of anxiety reduction, such as Aldridge, Levin, Seidler, & Slotkin (2005), who described serotonergic receptors dysfunction related to an anxiolytic response, when CPF was administered on PND 1 to 4, although in this study that was tested with another type of protocol. Pope et al. (1992) also found a decrease in locomotor activity after an acute dose of CPF, yet activity restored in a few days after behavioural evaluation. Dam, Seidler and Slotkin (2000) registered lower locomotor activity in rats treated with 1 mg/kg on PND 1 to 4. For their part, Carr et al. (2001) observed a reduction in locomotor activity postnatally (PND 1-5 and 7-21), though doses used were higher than ours. However, Sánchez-Amate et al. (2001) reported an anxiety increase, after acute CPF exposure, in the plus maze (rats were 90 days old). Levin et al. (2002) described locomotor hyperactivity in prenatally CPF exposed rats, as well as Grabovska & Salyha (2015) detected ADHD-like behaviour (hyperactivity). Probably, differences between studies are caused, mainly, by different behavioural protocols. We also should note that consequences in neurochemical systems, as GABAergic or serotonergic ones, may depend on the period of

time exposed to CPF. In that way, maybe effects on this system are mainly anxiolytic when exposure is critically carried out in postnatal stage. Although we only found treatment effect in the Visually-cued task, it seemed that, in any way, exposure on PND 10 to 15 was effective (though less than in other studies, probably due to the combination of a less vulnerable neurochemical system and a behavioural evaluation in the old age). It would be interesting to carry out a study with different times of CPF administration, keeping the spatial learning measurements in the old age, in order to be able to compare it with our results.

In respect of consequences on spatial learning, there already are studies in the scientific literature that proved a sexually-dimorphic effect of CPF on spatial learning. For example, in Gómez-Giménez et al. (2017), amongst rats exposed to CPF (and more pesticides), only CPF-treated male rats had a worse spatial learning performance than control male rats, while as CPF didn't affect performance of females compared to control female group. In fact, there was even an improvement in CPF-treated female rats when "exposed to 1mg/kg of chlorpyrifos", in reference memory measured through ARM. However, male performance was generally better than female one, as can be expected of the spatial learning. In this study, exposition was carried out from GD 7 to PND 21. For his part, Mamczarz et al. (2016) showed that only control male rats had a good performance when memory retention was measured, in a manner that CPF-treated male rats (prenatally exposed) performed as females. Moreover, Johnson et al. (2009) found that CPF only had negative effect when working memory performance of males was compared to the control males' one, when exposed postnatally from PND 1 to PND 21. Levin et al. (2002) described contradictory data, showing that only CPF-treated females were impaired, although we should note that, in this case, they were exposed in gestational stage (GD 17 – 20). In fact, a year before, Levin et al. (2001) found a better performance in female CPF exposed rats, whereas males had got a worse performance (PND 1-4). On the other hand, in Maurissen et al. (2000) there was almost no effect of CPF exposure from GD 6 to LD (lactation day) 6 at low doses (including 1 mg/kg), though pups evaluated weren't directly exposed to CPF in their PND (they received it by maternal milk).

It's clear that males, in general and biologically determined, have got a better spatial learning ability compared to the females, as it's summarized in Rahman and Koerting (2008). In addition, it also seems that there are a critical period in which CPF exposure have more or less effect in spatial cognition, and that this effect is not the same for males and females. Gómez-Gíménez et al. (2017) connected spatial learning and pro-inflammatory response in hippocampus after CPF exposure. They found an increase of pro-inflammatory interleukin B

in males exposed, but no in females. Moreover, there are multiple exogenous factors that can alter or disrupt the natural endocrine function, including the sexual hormones. In this sense, Maqbool, Mostafalou, Bahadar and Abdollahi (2016) talk about endocrine disrupting chemicals (EDC) that "are released into environment from different sources", and mention studies, such as Fent and Stegeman (1991), or Fent (2003), in which different xeonobiotics alter the steroid hormones system. In fact, Buratti et al. (2011) found disruption (by a deficit) of aromatase activity (which aromatize testosterone (TST) into estradiol) and an increase in TST metabolites (product of TST hydrolysis by TST hydroxylase hormone) in rats exposed both prenatally and postnatally to low doses of CPF, in absence of AChE inhibition.

Data exposed until now could suggest that CPF has got a masculinization effect, even when administered at doses not enough to induce ChE inhibition. In our study, control rats worsened their performance in spatial learning after the Transfer task (in which platform was removed), but we observed in descriptive statistics that this effect was evident, especially in females, yet there was no significant difference between sexes. Conversely, CPF-treated rats didn't show a decrease in their performance. We also should have in account the age of animals when they were evaluated: 14 months old, almost senectitude. It's likely that CPF possible effects dissipated when rats reached that old age, and possibly steroid hormones activity was completely restored at the beginning of spatial learning evaluation. However, females maybe were masculinized when they were exposed to CPF in synaptogenesis phase (remember that there are interdependence between endocrine and nervous systems); and we can also postulate a compensatory cholinergic mechanism (of course, not related to ChEs) that turned on in old age, which prevented the worse performance in CPF-exposed rats after the Transfer task. However, we should be cautious, as Levin et al. (2001) didn't found any masculinization effect or improvement in females spatial learning when rats were exposed from PND 11 to 14 (very similar to the ours), although these effects emerged when rats were challenged with scopolamine. In order to confirm what was said, studies with a longer number of animals by sex and with measures of steroid hormones and aromatase should be developed. Moreover, in these studies spatial learning should be measured several times during the life of animals, so that we could verify the importance of the age and its relation with an early CPF exposure. It also would be interesting to introduce several administrations in different points of time, like cohorts.

5. CONCLUSIONS

Further research is needed to prove effects of CPF in mammal neurodevelopment. Although our data doesn't disagree with other provided by scientific literature, it's evident that, at least, negative consequences, when measured in old age, aren't as impairing as they could be in youth animals. Moreover, behavioural measures should be complemented with physiological data, in order to explain the cause of CPF-induced neurobehavioural deficits. In our case, it can't be said that we found deficits: given that we found an absence of decrease in spatial learning performance, and a mild locomotor hypoactivity, we should even say that CPF enhanced performance. Additional studies could account for this effects, and tell us if there is any compensatory, long-term neurochemical mechanism that, either due or not, to CPF, underlies this data. Furthermore, we think that investigation on the relation between cholinergic and other neurochemical/hormonal systems will clarify the issue, giving us explanations alternative to those exclusively related to ACh metabolism and activity.

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APPENDIX A: Physical-chemical characteristics of chlorpyrifos (CPF).

Chemical characteristics of clorpyrifos	
Chemical name	CAS: *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate ISO: CHLORPYRIFOS (ing.), CLORPIRIFOS (sp.)
Chemical formula	$C_9H_{11}Cl_3NO_3PS$
Molecular weight	350,57 g/mol
Melting point	41-43,5 ºC
Boiling point	~ 160 ºC
Vapor pressure at 25ºC	0,0025 Pa
Water solubility at 25ºC	2 mg/L
Organic solvent solubility	79% w/w in isooctane. 43% w/w in methanol
Relative density (g/mL)	1,398 (at 43,5 ºC)

APPENDIX B: Characteristics of main studies about CPF exposure and spatial learning in MWM

Age of exposure		Spatial learning assessment	Dosification	Article
Prenatal		Post-natal days (PND) 40-45	25 mg/kg/day x 10 days (s.c.)	Mullins, Xu, Pereira, Prescrille et al. (2015)
		PND 38	25 mg/kg/day x 10 days (s.c.)	Mamczarz et al. (2016)
		1-2 months after CPF exposure	1 mg/kg/day (GD 7 – PND 0)	Gómez-Giménez et al. (2017)
Posnatal	Early	PND 24	0.3/7 mg/kg (s.c.) Pre-weaning: PND 7, 11 and 15. Post-weaning: PND 24, 26 and 28.	Jett, Navoa, Beckles & McLemore (2001)
		4 and a half months after CPF exposure	10/15 mg/kg/day x 30 days (s.c.)	Terry, Beck, Warner, Vandenhuerk & Callahan (2012)
		1-2 months after CPF exposure	1 mg/kg/day (PND 0- 21)	Gómez-Giménez et al. (2017)
		11 months after exposure	300 mg/kg (s.c.)	Mullins, Xu, Pereira, Mamczarz et al. (2013)
	Adult	1 or 14 days after CPF exposure	2,5-100 mg/kg (s.c.)	Terry et al. (2003)
		22 weeks after the second CPF exposure	166/250 mg/kg	Sánchez-Santed, Cañadas, Flores, López-Grancha & Cardona (2004)
		21 weeks after CPF exposure	2 x 250 mg/kg (s.c.)	Cañadas, Cardona, Dávila & Sánchez-Santed (2005)
		16 hours after CPF exposure	1, 5 o 10 mg/kg (gavage)	Yan et al. (2012)
		72 hours/22 weeks after CPF exposure	25 mg/kg/día x 10 days (s.c.)	López-Granero, Cardona, Giménez, Lozano, Barril, Aschner et al. (2014)
		7 months after CPF exposure	5 mg/kg/day x 6 meses (diet)	López-Granero, Ruiz-Muñoz et al. (2016)
¿? (non-specified)		3 days after CPF exposure	250 mg/kg (s.c.)	López-Granero, Cañadas et al. (2013)
		21 weeks after CPF exposure	5 mg/kg/day x 31 weeks (diet)	López-Granero, Cardona, Giménez, Lozano, Barril, Sánchez-Santed et al. (2013)

APPENDIX C: Characteristics of other studies using ARM to evaluate spatial learning after CPF exposure.

Age of exposure		Spatial learning assessment	Dosification	Article
Prenatal		PND 21	1 / 5 mg/kg/day x 4 days	Levin et al. (2002)
Posnatal	Temprana	PND 29-60	Progressive (1 to 6 mg/kg/day x 21 days (gavage))	Johnson, Chambers, Nail, Givaruangsawat & Carr (2009)

APPENDIX D: Control variables data.

Table A1: *Effects of each variable on total distance moved in the Spatial learning task.*

	FACTOR	F	Deg. of freedom	Sig.
Between-subjects	TREATMENT	0.107	1	0.747
	SEX	1.375	1	0.251
	Error		1	
Within-subjects	TRIAL	36.593	1.980	**0.000**
	Error (TRIAL)		53.447	
	SESSION	23.980	7	**0.000**
	Error (SESSION)		189	
Interactions	TREATMENT * SEX	1.311	1	0.262
	TRIAL * TREATMENT	0.747	1.980	0.477
	TRIAL*SEX	0.123	1.980	0.883
	TRIAL*TREATMENT * SEX	0.618	1.980	0.541
	SESSION * TREATMENT	0.604	7	0.752
	SESSION*SEX	3.144	7	**0.004**
	SESSION*TREATMENT* SEX	0.344	7	0.933
	TRIAL*SESSION	3.174	10.346	**0.001**
	TRIAL*SESSION*TREATMENT	0.445	10.346	0.928
	TRIAL*SESSION*SEX	1.148	10.346	0.325
	TRIAL*SESSION * TREATMENT* SEX	0.950	10.346	0.490
	ERROR (TRIAL * SESSION)		279.337	

Note: The accepted level of significance for analyses was $p \leq 0{,}05$.

Table A2: *Effect of each variable on velocity (mean) in the Spatial learning task.*

	FACTOR	F	Deg. of freedom	Sig.
Between-subjects	TREATMENT	3.951	1	0.057
	SEX	0.206	1	0.654
	Error		27	
Within-subjects	TRIAL	21.281	1.975	**0.000**
	Error (TRIAL)		53.325	
	SESSION	9.903	4.768	**0.000**
	Error (SESSION)		128.735	
Interactions	TREATMENT * SEX	0.001	1	0.975
	TRIAL * TREATMENT	0.455	1,975	0.634
	TRIAL*SEX	1.289	1.975	0.284
	TRIAL*TREATMENT * SEX	1.702	1.975	0.192
	SESSION * TREATMENT	0.900	4.768	0.480
	SESSION*SEX	1.369	4.768	0.242
	SESSION*TREATMENT* SEX	2.029	4.768	0.082
	TRIAL*SESSION	6.358	11.138	**0.000**
	TRIAL*SESSION*TREATMENT	0.540	11.138	0.877
	TRIAL*SESSION*SEX	1.442	11.138	0.152
	TRIAL*SESSION * TREATMENT* SEX	1.341	11.138	0.200
	ERROR (TRIAL * SESSION)		300.737	

Note: The accepted level of significance for analyses was $p \leq 0,05$.

Table A3: *Effects of between-subject variables on total distance moved in the Transfer task.*

FACTOR	F	Deg. of freedom	Sig.
TREATMENT	0.237	1	0.630
SEX	0.396	1	0.534
TREATMENT * SEX	0.020	1	0.888
Error		27	

Note: The accepted level of significance for analyses was $p \leq 0,05$.

Table A4: *Effects of between-subject variables on velocity (mean) in the Transfer task.*

FACTOR	F	Deg. of freedom	Sig.
TREATMENT	0.557	1	0.462
SEX	0.211	1	0.650
TREATMENT * SEX	0.111	1	0.742
Error		27	

Note: The accepted level of significance for analyses was $p \leq 0,05$.

Table A5: *Effects of each variable on total distance moved in the Reinstating memory task compared to the last session of Spatial learning task.*

	FACTOR	F	Deg. of freedom	Sig.
Between-subjects	TREATMENT	0.952	1	0.338
	SEX	0.007	1	0.933
	Error		27	
Within-subjects	TRIAL	2.599	3	0.058
	Error (TRIAL)		81	
	SESSION	1.402	1	0.247
	Error (SESSION)		27	
Interactions	TREATMENT * SEX	0.033	1	0.858
	TRIAL * TREATMENT	0.368	3	0.776
	TRIAL*SEX	0.249	3	0.862
	TRIAL*TREATMENT * SEX	0.963	3	0.415
	SESSION * TREATMENT	3.847	1	0.060
	SESSION*SEX	0.495	1	0.488
	SESSION*TREATMENT* SEX	1.016	1	0.322
	TRIAL*SESSION	0.870	2.297	0.437
	TRIAL*SESSION*TREATMENT	0.548	2.297	0.605
	TRIAL*SESSION*SEX	2.048	2.297	0.131
	TRIAL*SESSION * TREATMENT* SEX	0.275	2.297	0.790
	ERROR (TRIAL * SESSION)		62.030	

Note: The accepted level of significance for analyses was $p \leq 0,05$.

Table A6: *Effects of each variable on velocity (mean) in the Reinstating memory task compared to the last session of Spatial learning task.*

	FACTOR	F	Deg. of freedom	Sig.
Between-subjects	TREATMENT	1.446	1	0.240
	SEX	0.308	1	0.583
	Error		27	
Within-subjects	TRIAL	1.354	3	0.263
	Error (TRIAL)		81	
	SESSION	1.906	1	0.179
	Error (SESSION)		27	
Interactions	TREATMENT * SEX	0.403	1	0.531
	TRIAL * TREATMENT	0.901	3	0.444
	TRIAL*SEX	2.628	3	0.056
	TRIAL*TREATMENT * SEX	0.924	3	0.433
	SESSION * TREATMENT	0.042	1	0.839
	SESSION*SEX	0.143	1	0.709
	SESSION*TREATMENT* SEX	0.276	1	0.603
	TRIAL*SESSION	1.462	3	0.231
	TRIAL*SESSION*TREATMENT	0.886	3	0.452
	TRIAL*SESSION*SEX	4.427	3	**0.006**
	TRIAL*SESSION * TREATMENT* SEX	1.334	3	0.269
	ERROR (TRIAL * SESSION)		81	

Note: The accepted level of significance for analyses was $p \leq 0{,}05$.

Table A7: *Effect of each variable on total distance moved in Reversal task.*

	FACTOR	F	Deg. of freedom	Sig.
Between-subjects	TREATMENT	1.181	1	0.287
	SEX	0.434	1	0.516
	Error		27	
Within-subjects	TRIAL	15.698	2.255	**0.000**
	Error (TRIAL)		60.890	
	SESSION	4.360	2	**0.018**
	Error (SESSION)		54	
Interactions	TREATMENT * SEX	0.115	1	0.737
	TRIAL * TREATMENT	0.420	2.255	0.683
	TRIAL*SEX	1.829	2.255	0.165
	TRIAL*TREATMENT * SEX	0.648	2.255	0.545
	SESSION * TREATMENT	0.086	2	0.918
	SESSION*SEX	0.673	2	0.514
	SESSION*TREATMENT* SEX	1.402	2	0.255
	TRIAL*SESSION	0.887	3.722	0.469
	TRIAL*SESSION*TREATMENT	0.783	3.722	0.531
	TRIAL*SESSION*SEX	0.680	3.722	0.597
	TRIAL*SESSION * TREATMENT* SEX	1.428	3.722	0.233
	ERROR (TRIAL * SESSION)		100.506	

Note: The accepted level of significance for analyses was $p \leq 0{,}05$.

Table A8: *Effect of each variable on velocity (mean) in Reversal task.*

	FACTOR	F	Deg. of freedom	Sig.
Between-subjects	TREATMENT	0.840	1	0.368
	SEX	0.012	1	0.913
	Error		27	
Within-subjects	TRIAL	1.766	2.267	0.175
	Error (TRIAL)		61.198	
	SESSION	2.966	2	0.060
	Error (SESSION)		54	
Interactions	TREATMENT * SEX	0.154	1	0.698
	TRIAL * TREATMENT	0.434	2.267	0.675
	TRIAL*SEX	0.157	2.267	0.879
	TRIAL*TREATMENT * SEX	0.729	2.267	0.503
	SESSION * TREATMENT	0.379	2	0.261
	SESSION*SEX	0.382	2	0.685
	SESSION*TREATMENT* SEX	0.772	2	0.467
	TRIAL*SESSION	1.142	4.324	0.341
	TRIAL*SESSION*TREATMENT	2.164	4.324	0.072
	TRIAL*SESSION*SEX	0.246	4.324	0.923
	TRIAL*SESSION * TREATMENT* SEX	1.977	4.324	0.097
	ERROR (TRIAL * SESSION)		116.745	

Note: The accepted level of significance for analyses was $p \leq 0{,}05$.

Table A9: *Effect of each variable on total distance moved in Visually-cued task.*

	FACTOR	F	Deg. of freedom	Sig.
Between-subjects	TREATMENT	0.867	1	0.360
	SEX	1.891	1	0.180
	Error		27	
Within-subjects	TRIAL	2.901	3	**0.040**
	Error (TRIAL)		81	
	SESSION	4.150	2	**0.021**
	Error (SESSION)			
Interactions	TREATMENT * SEX	2.918	1	0.099
	TRIAL * TREATMENT	0.230	3	0.876
	TRIAL*SEX	0.915	3	0.437
	TRIAL*TREATMENT * SEX	0.535	3	0.660
	SESSION * TREATMENT	1.711	2	0.190
	SESSION*SEX	0.189	2	0.828
	SESSION*TREATMENT* SEX	0.274	2	0.762
	TRIAL*SESSION	8.435	3.986	0.000
	TRIAL*SESSION*TREATMENT	0.303	3.986	0.875
	TRIAL*SESSION*SEX	0.581	3.986	0.677
	TRIAL*SESSION * TREATMENT* SEX	1.798	3.986	0.135
	ERROR (TRIAL * SESSION)		107.627	

Note: The accepted level of significance for analyses was $p \leq 0{,}05$.

Table A10: *Effect of each variable on velocity (mean) in Visually-cued task.*

FACTOR		F	Deg. of freedom	Sig.
Between-subjects	TREATMENT	5.562	1	**0.026**
	SEX	0.910	1	0.349
	Error		27	
Within-subjects	TRIAL	3.222	3	**0.027**
	Error (TRIAL)		81	
	SESSION	4	1.620	**0.033**
	Error (SESSION)		43.727	
Interactions	TREATMENT * SEX	0.004	1	0.950
	TRIAL * TREATMENT	0.098	3	0.961
	TRIAL*SEX	0.824	3	0.485
	TRIAL*TREATMENT * SEX	0.884	3	0.453
	SESSION * TREATMENT	0.060	1.620	0.910
	SESSION*SEX	0.029	1.620	0.949
	SESSION*TREATMENT* SEX	0.170	1.620	0.799
	TRIAL*SESSION	0.285	3.983	0.887
	TRIAL*SESSION*TREATMENT	1.007	3.983	0.407
	TRIAL*SESSION*SEX	0.602	3.983	0.661
	TRIAL*SESSION * TREATMENT* SEX	0.266	3.983	0.898
	ERROR (TRIAL * SESSION)		107.534	

Note: The accepted level of significance for analyses was $p \leq 0{,}05$.

APPENDIX E: Control variables graphics.

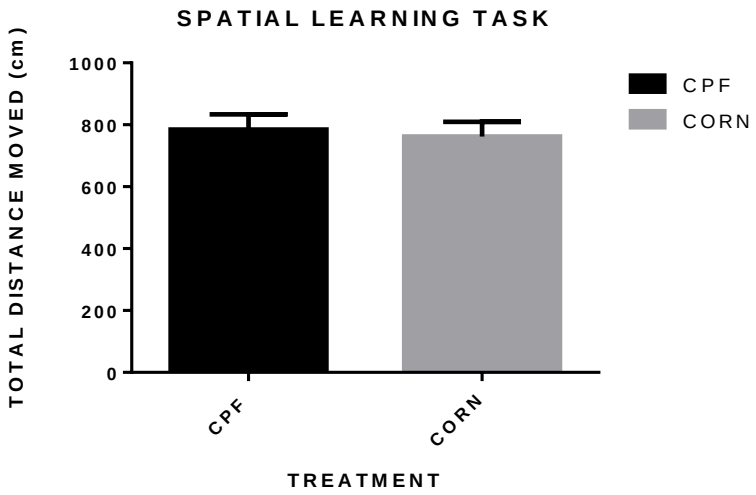

Figure B1: Total distance moved (cm) by each treatment group in Spatial learning task.

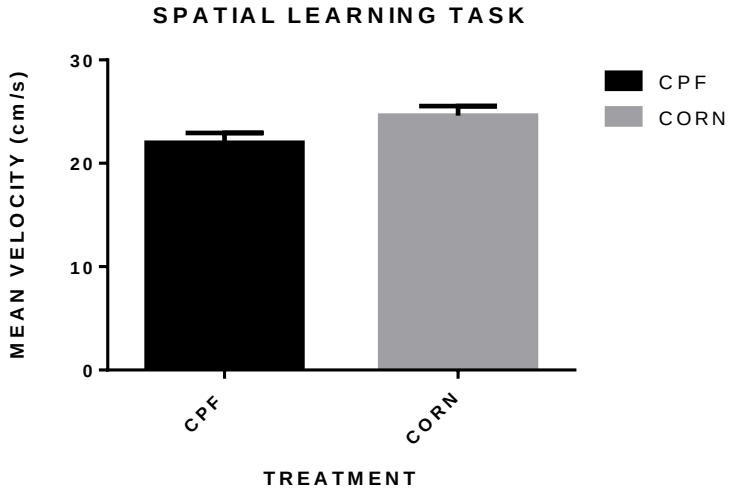

Figure B2: Mean velocity (cm/s) by each treatment group in Spatial learning task.

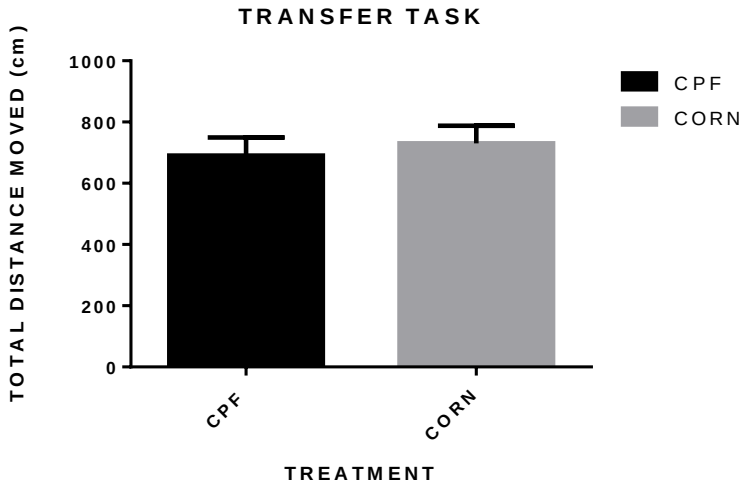

Figure B3: Total distance moved (cm) by each treatment group in Transfer task.

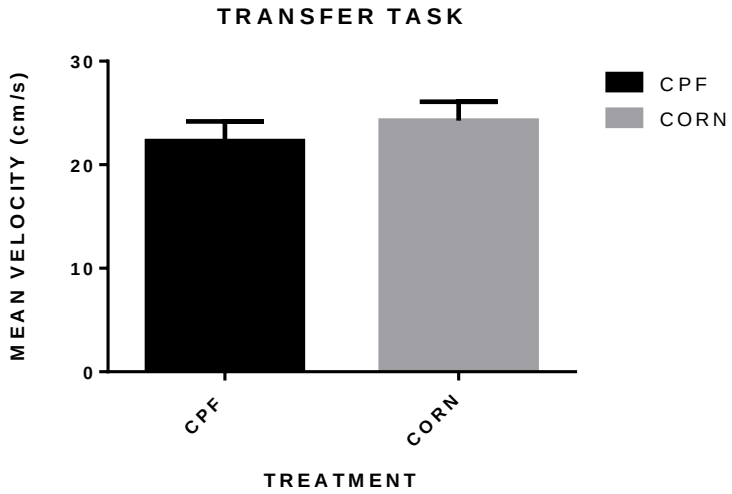

Figure B4: Mean velocity (cm/s) by each treatment group in Transfer task..

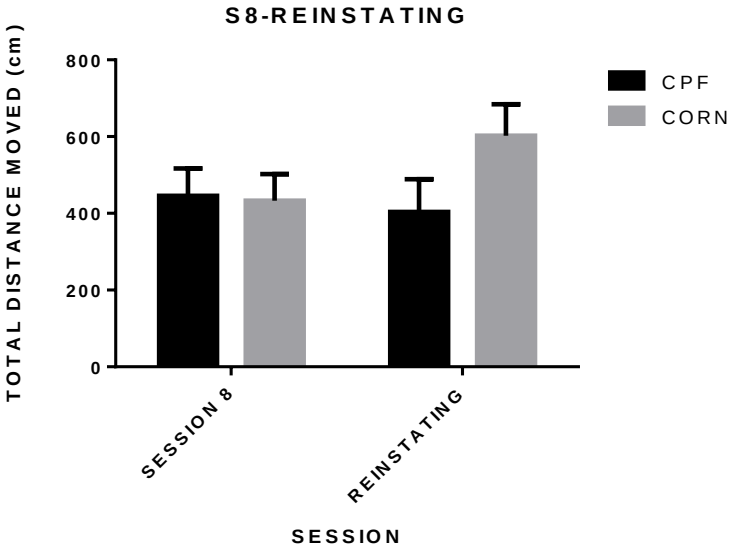

Figure B5: Total distance moved (cm) by each treatment group in the last session of Spatial learning task (S8) compared to the Reinstating memory task.

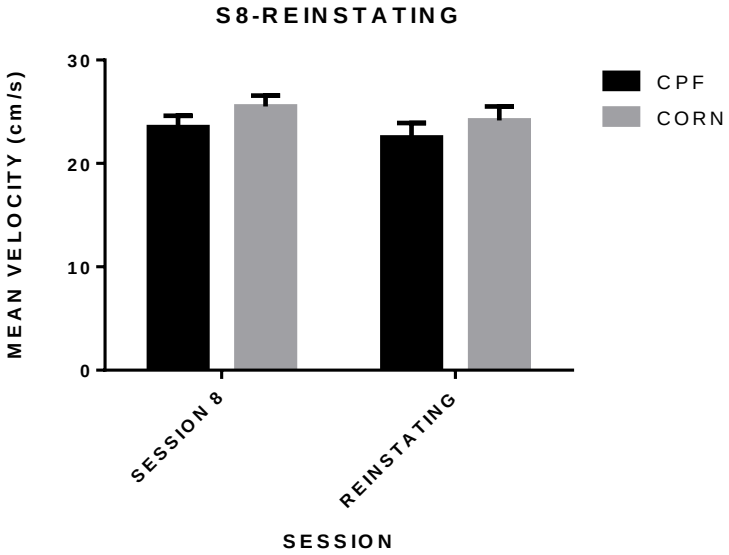

Figure B6: Mean velocity (cm/s) by each treatment group in the last session of Spatial learning task (S8) compared to the Reinstating memory task.

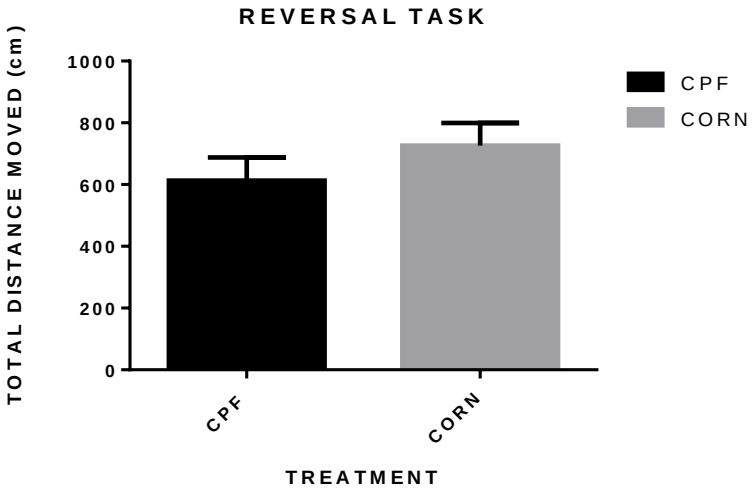

Figure B7: Total distance moved (cm) by each treatment group in Reversal group.

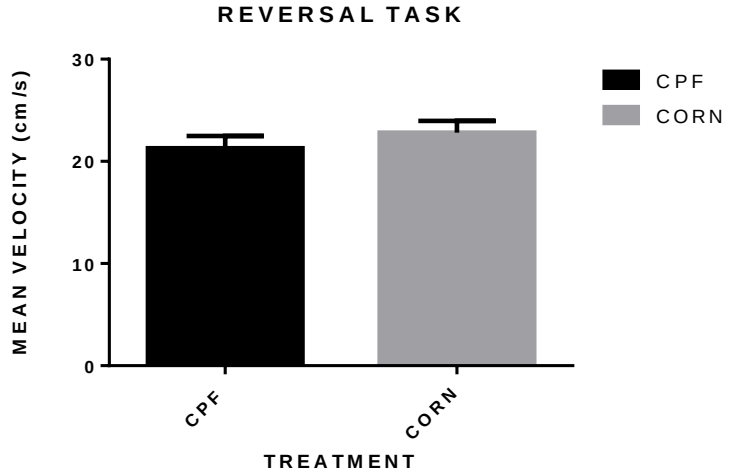

Figure B8: Mean velocity (cm/s) by each treatment group in Reversal task..

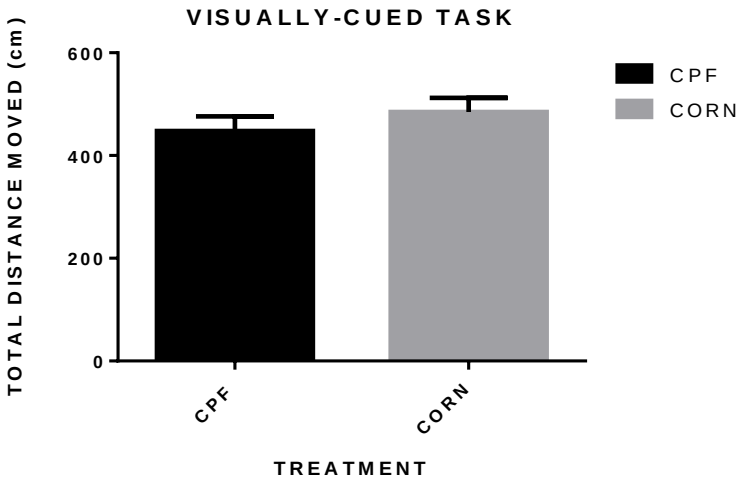

Figure B9: Total distance moved (cm) by each treatment group in Visually-cued task.