

**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL  
INSTITUTO DE BIOCÊNCIAS  
BACHARELADO EM BIOTECNOLOGIA**

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**PAPEL DA METILAÇÃO DE HISTONAS H3 NOS EFEITOS  
TOXICOLÓGICOS DO METILMERCÚRIO E/OU PALMITATO DE RETINOL  
EM RATOS WISTAR**

**Porto Alegre**

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Trabalho de Conclusão de curso  
apresentado como requisito parcial para  
obtenção do título de Bacharel em  
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Orientador: Prof. Dr. José Cláudio Fonseca  
Moreira

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*“If you are losing your soul and you know it, then you’ve still got a soul left to lose”*

*- Charles Bukowski*

## AGRADECIMENTOS

Impossível não começar pela minha família. Meu pai e minha mãe, ALEXANDRE e DENISE, são ambos professores e nunca me negaram uma resposta da incansável pergunta: *mas porquê?* Porque o céu é azul? Porque as pessoas morrem? Porque os dinossauros não existem mais? Esses eram questionamentos que tranquilamente seriam feitos pelo Flávio criança em um dia. Assim se estabelece um cientista. De certa forma me faz pensar que todos nascemos afeitos ao método científico, porém nem todos têm o privilégio de mantê-lo vivo. Se um pouco de mim ainda vibra nesse sentimento, devo isso aos meus pais, à minha criação. **Amo muito vocês.**

Se cheguei aqui, um pouco se deve à pessoa que me tornei. Contudo, isso tem pouco mérito meu e muito das pessoas que me cercam. Somos fruto do nosso entorno, a existência é compartilhada. Agradeço à minha irmã JÚLIA, pelo carinho e cuidado, pelo amor maternal. Agradeço aos meus amigos de infância, aqui citados FRANCISCO e MIGUEL, pelas infinitas risadas e companheirismo. Agradeço à DALVA, minha outra mãe, quem me mostrou que o mundo está longe do meu umbigo; quem me tornou mais humilde. Agradeço aos meus colegas e amigos da Biotecnologia, por terem suavizado o meu dia-a-dia de graduação, pareceu fácil. Agradeço a todos do laboratório 32, por me mostrarem que o ambiente de trabalho pode ser leve; por nunca me negarem ajuda ou algum ensinamento. Agradeço todos os dias, quem não sai de perto de mim: meus Orixás. Eu agradeço ao sítio, eu agradeço à vila. Se como no momento em que escrevo, alguém lê e sente seu coração apertado: saiba que sempre irei te abastecer com a minha militante amizade e o meu atento amor.

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*“Um bom orientador é aquele que você pode rir junto”*

*- Carlos Termignoni*

Foi assim que nasceu o laboratório 32 para mim, ou eu para ele. Zé Cláudio de início me respeitou como pessoa e demonstrou que eu estava livre para conduzir um projeto próprio. Hoje posso dizer ao Termignoni: encontrei um lugar sadio que se preocupa em fazer ciência de verdade e também um *bom orientador*.

Novamente: MUITO OBRIGADO!

## INTRODUÇÃO

A contaminação de efluentes com mercúrio inorgânico geralmente é oriunda da mineração de ouro<sup>1; 2; 3; 4; 5</sup> ou pela indústria, como a produção de cloro e acetaldeído<sup>6; 7</sup>. Em rios, lagos, oceanos e solo o mercúrio inorgânico é transformado em seu derivado mais perigoso, o metilmercúrio (MeHg), por diversas bactérias redutoras de enxofre, redutoras de ferro, metanogênicas e membros da família *Geobacteraceae*<sup>6; 8; 9; 10</sup>. A principal via de contato com o MeHg ambientalmente é pelo consumo de peixes e crustáceos<sup>2; 11</sup>. Após o consumo, o MeHg deposita-se no fígado e nos rins e acaba afetando principalmente o sistema nervoso central<sup>12</sup>. Além disso, o MeHg é um poluente amplamente distribuído, tendo sido amostrado em localidades como Colômbia<sup>13</sup>, Brasil<sup>1</sup>, Guiana Francesa<sup>14</sup>, Europa<sup>15; 16</sup>, Taiwan<sup>17</sup> e Canadá<sup>18</sup>.

Diversos mecanismos que promovem a neurotoxicidade do MeHg tem sido propostos, como (i) o rompimento da homeostase de cálcio, (ii) indução de dano oxidativo e mal funcionamento da mitocôndria, (iii) interações com grupos sulfidris, (iv) captação de glutamato, (v) alteração da organização do citoesqueleto e outros<sup>6; 19; 20; 21; 22; 23; 24</sup>. A contaminação com MeHg durante a gravidez pode causar problemas no neurodesenvolvimento e déficits cognitivos em crianças<sup>25; 26; 27</sup>. Segundo trabalhos anteriores do nosso grupo de pesquisa, a exposição ao MeHg durante a gestação foi capaz de afetar a sinalização celular no hipocampo<sup>21</sup> e cerebelo<sup>28</sup>.

Os efeitos tóxicos de substâncias químicas nos organismos não estão restritos apenas à exposição de xenobióticos. Em doses elevadas, moléculas essenciais como vitaminas podem ser danosas à saúde; dentre elas, está a vitamina A. A vitamina A é um metabólito essencial para o desenvolvimento adequado do organismo. A ausência de vitamina A na dieta é um problema para alguns países em desenvolvimento e pode causar danos sérios à saúde, inclusive a cegueira<sup>29</sup>. Contudo, o consumo excessivo da vitamina também é prejudicial à saúde, tendo inclusive efeitos teratogênicos<sup>30; 31</sup>. Devido ao crescente consumo de alimentos fortificados com vitaminas, pílulas suplementares, peixes suplementados com vitamina A e outras fontes de vitamina A, é comum se atingir concentrações elevadas, porém não-teratogênicas. Além disso, existem recomendações de suplementação de vitamina A em grávidas<sup>32; 33</sup>. Um trabalho anterior do nosso grupo de pesquisa determinou que a suplementação de uma dose considerada segura de vitamina A durante a gravidez causa desvios comportamentais e estimula dano oxidativo na prole<sup>34</sup>.

A exposição humana a diversos estressores tem ganhado destaque nos últimos anos. Devido à crescente exposição potencial ao MeHg e à vitamina A, o foco do presente

trabalho visa justamente à avaliação dos efeitos isolados e combinados desses compostos. A exposição durante a gestação e lactação ao MeHg e palmitato de retinol (VitA) demonstrou ser capaz de alterar parâmetros neurocomportamentais, oxidativos e toxicológicos na prole<sup>35; 36</sup>. Com isso, os dois compostos desempenham um papel toxicológico no desenvolvimento da prole, levantando a hipótese de que os efeitos possam estar sendo passíveis de herança transgeracional e modulados epigeneticamente.

Apesar do termo *epigenética* ter sofrido mudanças de significado ao longo do tempo<sup>37</sup>, hoje está relacionado ao estudo de alterações da função gênica que além de serem passíveis de herança também são independentes de DNA, mais especificamente sua sequência nucleotídica<sup>38</sup>. Existem diversos mecanismos que são considerados epigenéticos, dentre os mais aceitos e estudados estão três: (i) modificações de DNA, como metilação, formilação, hidroximetilação de bases nitrogenadas; (ii) modificações de histonas, como metilação, acetilação, fosforilação; (iii) expressão de RNAs não-codificantes, como microRNAs, RNAs de interferência, RNAs longos<sup>39</sup>.

O ácido retinóico, é geralmente conhecido como vitamina A, é importante para morfogênese e diferenciação de células<sup>40; 41; 42</sup>. Já se demonstrou que este composto afeta o perfil de metilação de DNA, causa modificações em histonas e altera a expressão de RNAs não-codificantes<sup>43</sup>. Por outro lado, MeHg também foi capaz de alterar padrões de metilação de histonas<sup>44</sup> e causar efeitos toxicológicos em modelos transgeracionais de peixe zebra (*Danio rerio*)<sup>45</sup>. Ademais, a coexposição de MeHg e cádmio durante a gestação também foi capaz de desencadear efeitos transgeracionais em camundongos, como alteração da tolerância à glicose e da massa de tecido adiposo<sup>46</sup>. Dessa forma, a o conjunto de estudos científicos aponta para uma possível modulação epigenética tanto do MeHg quanto da vitamina A. Se torna importante a avaliação desses possíveis efeitos após exposições em ratos que representem cenários reais de contaminação. O presente trabalho justamente pretende determinar se tais exposições, a vitamina A e MeHg, são capazes de alterar perfis epigenéticos e prejudicar a saúde de futuras gerações.

## **Objetivos:**

### **Geral**

Determinar se a coexposição a vitamina A e MeHg é capaz de modular perfis epigenéticos e comprometer o desenvolvimento e a saúde de futuras gerações.

### **Específicos**

- 1) Determinar os efeitos da coexposição de VitA e MeHg sobre parâmetros de desenvolvimento, como preferência olfatória pela maravalha materna e tempo de abertura dos olhos na F2
- 2) Avaliar o efeito da coexposição (MeHg + VitA) sobre a massa dos órgãos internos e frequência de micronúcleos, em F2
- 3) Compreender o papel da VitA e MeHg na trimetilação de histonas 3 no cerebelo de ratos Wistar, tanto na F1 quanto na F2



**Co-exposure to methylmercury and retinyl palmitate during gestation and breastfeeding changes H3 epigenetic pattern and perpetuates transgenerational inheritance effects in Wistar rats**

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CEUA, Usage of Animals Ethics Commission; PND, postnatal day; GD, gestation day; VitA, retinyl palmitate; RAE, retinol activity equivalents; MeHg, methylmercury II chloride; MeHg+VitA, co-exposure of methylmercury and retinyl palmitate; F0, exposed pregnant females; F1, first generation; F2 second generation; CN, control; PCE, polychromatic erythrocytes cells; NCE, normochromatic erythrocytes cells; MNPCE, micronuclei frequencies per 2000 PCEs; H3, histone 3

1 **ABSTRACT**

2           Methylmercury (MeHg) is a pollutant present in fish, being seafood consumption  
3 the main route of contamination. It was already demonstrated the MeHg's capability to  
4 cause neurodevelopment impairments and cognitive deficits in offspring and children  
5 after gestation exposure. Vitamin A, a fish micronutrient, is an essential molecule, but  
6 high doses can lead to cognitive dysfunctions and even teratogenesis. Moreover, vitamin  
7 A is also recommended for supplementation on pregnant. Doses considered low for both  
8 MeHg and vitamin A can establish toxicological effects and influence histone  
9 modifications, DNA methylation and non-coding RNAs expression. Besides, these  
10 effects seem to be perpetuated by transgenerational inheritance. This work consisted of  
11 administrating low doses of MeHg (0.5mg/kg/day) and/or retinyl palmitate (VitA, 7500  
12 retinol activity equivalents/kg/day) to Wistar rats (F0) during gestation and lactation. It  
13 evaluates the effects in subsequent generations (F1 and F2). The exposures were capable  
14 to influence neurobehavior, organs weight, cytogenetic damage and histone methylation  
15 until F2. MeHg and VitA interaction in co-exposure group is conflicting, in eye-opening  
16 time, a neurobehaviour test, the effect appeared to be synergistic. However, the organs  
17 weight of the co-exposure group was at control level in nearly all parameters. This work  
18 is the very first to determine the transgenerational inheritance of toxicological effects of  
19 environmental co-exposure to MeHg and/or VitA. Thus, these compounds are capable to  
20 induce health injury and epigenetic modulations of histone H3. Our data suggests that  
21 populations in high exposed areas to MeHg and/or VitA may be affected for more than  
22 one generation once exposure has finished.

23 **Keywords: methylmercury; vitamin A; transgenerational inheritance; histone 3;**  
24 **epigenetics; environmental toxicology;**

25

## 26 **1. Introduction**

27 In the course of life, we are exposed to a series of toxicants, which cause damage to  
28 the central nervous system, such as methylmercury (MeHg). Also, other substances  
29 become toxic in higher doses causing congenital problems. An example of this is vitamin  
30 A (Ritchie et al., 1998; Schnorr et al., 2011a; Schnorr et al., 2015). However, little is  
31 known about the possible effects of these substances over the generations.

32 Contamination of effluents, inorganic mercury is transformed by reducing bacteria  
33 (Si et al., 2015) into its most dangerous derivative: MeHg. With high-absorption rates of  
34 MeHg, a biomagnification process is started (Marrugo-Negrete et al., 2018) through fish  
35 and crustaceans consumption (Bourdineaud et al., 2011; Marrugo-Negrete et al., 2013).  
36 Once absorbed, MeHg is transported into the bloodstream, deposits in the kidneys and  
37 liver and affects mainly the central nervous system (Santos-Lima et al., 2020). Hence,  
38 MeHg represents a serious risk to public health. Traditional communities in the Brazilian  
39 Amazon region were already affected (Vega et al., 2018; Hacon et al., 2020; Santos-Lima  
40 et al., 2020). Besides the region has higher concentrations of inorganic Hg naturally, there  
41 are also decades of improper gold mining companies-waste accumulated (Vieira et al.,  
42 2018). MeHg penetrates the placenta and the blood-brain barrier of the fetuses (Crespo-  
43 Lopez et al., 2009; Oliveira et al., 2015). Chronic exposure in low concentrations can  
44 trigger impaired motor and cognitive development and psychological disorders in  
45 offspring (Llop et al., 2017a; Llop et al., 2020).

46 Pregnant and children's health is also susceptible to other diet-related nutrients,  
47 especially vitamin A. Vitamin A is essential for embryonic development, metabolic cell  
48 differentiation, and physiological parameters (Howson et al., 1998). Levels of  
49 supplementation of vitamin A defined by Recommended Dietary Allowances (RDA,  
50 1989) during pregnancy and lactation ranges from 400 to 500 retinol activity equivalents

51 (RAE). However, these levels are often exceeded considering contemporaneous  
52 alimentation. Consumption of high-absorptive vitamin A derivatives such as preformed  
53 vitamin A (Bauernfeind, 1972) is the main intake of vitamin A in the Europe and United  
54 States (Olson, 1996). Present in enriched foods, fish oil and supplementation pills the  
55 consumption of preformed vitamin A during pregnancy in levels above the recommended  
56 can lead to teratogenesis and congenital defects in the fetus (Azais-Braesco and Pascal,  
57 2000).

58 Retinoids demonstrated to have pro-oxidant characteristics, in rats supplemented with  
59 non-teratogenic doses (Azais-Braesco and Pascal, 2000; Allen and Haskell, 2002; da  
60 Rocha et al., 2010). Among some of its effects, in therapeutic doses resulted in  
61 compromised liver and kidney redox balance in mothers and their children (Ross, 2010;  
62 Li and Marikawa, 2020). Besides, evidence suggests that exposure to MeHg also impact  
63 redox (im)balance, causing: ROS production, glutathione depletion (GSH), excessive  
64 calcium accumulation ( $\text{Ca}^{2+}$ ), and decreased mitochondrial membrane potential in the  
65 nervous and immune systems (Caballero et al., 2017).

66 Despite presenting well-documented studies on MeHg and VitA, there is a lack of  
67 information on the impacts resulting from their combined exposure. Furthermore, there  
68 is a trending demand to discover their effects over the generations once both showed to  
69 influence the health of offspring (Huang et al., 2011; Schnorr et al., 2011a). Thus, it is  
70 necessary to understand the effects of co-exposure of vitamin A and MeHg as they are  
71 environmentally available molecules during the prenatal period and to investigate the  
72 possible consequences throughout the life of the offspring. Wherefore, this study aims to  
73 (i) evaluate the epigenetic and toxicological parameters of offspring whose mothers, F1,  
74 or grandmothers, F2, were exposed to low doses of MeHg with or without vitamin A and

75 their descendants and (ii) determine if these exposures trigger transgenerational  
76 inheritance.

## 77 **2. Materials and Methods**

78 All practices involving animals in this work respected the Principles of Laboratory  
79 Animal Care, determined by the US National Research Council (NRC, 2011). Moreover,  
80 all procedures also followed the Brazilian guidelines, being homologated by the Usage of  
81 Animals Ethics Commission (CEUA) of the Universidade Federal do Rio Grande do Sul  
82 under the number of 34886.

### 83 **2.1. Animals conditions**

84 Wistar rats (*Rattus norvegicus*) were housed in polypropylene cages at a 12h light  
85 cycle and controlled temperature (22°C). The access to water and standard commercial  
86 chow (CR1 Lab Chow, Nuvilab, Curitiba, Brazil) was *ad libitum*. Using vaginal smear,  
87 the estrous cycle from nulliparous females was evaluated (Marcondes et al., 2002).  
88 Females in proestrus or estrus were considered as sexually receptive and caged with  
89 paired males (90 days old, PND90) in a proportion of 1:1. On the next day, females with  
90 traces of sperm in the vaginal lavage were considered to be in gestation day 0 (GD0). The  
91 treatments were administered chronically from GD0 until postnatal day 21 (PND21),  
92 totalizing 42 days.

93 Pregnant females were assigned to groups randomly. The delivery day was defined  
94 as postnatal day 1 (PND1), the number and weight of pups were accompanied. On  
95 PND10, pups were sexed and immediately separated in PND21, at the end of the lactation.  
96 After the end of lactation period, no further treatment was administered to dams neither  
97 offspring.

### 98 **2.2. Exposures**

99 Exposures followed the same protocol of previously published works by our research  
100 group (Espitia-Perez et al., 2018a; Espitia-Perez et al., 2018b). Briefly, a stock solution

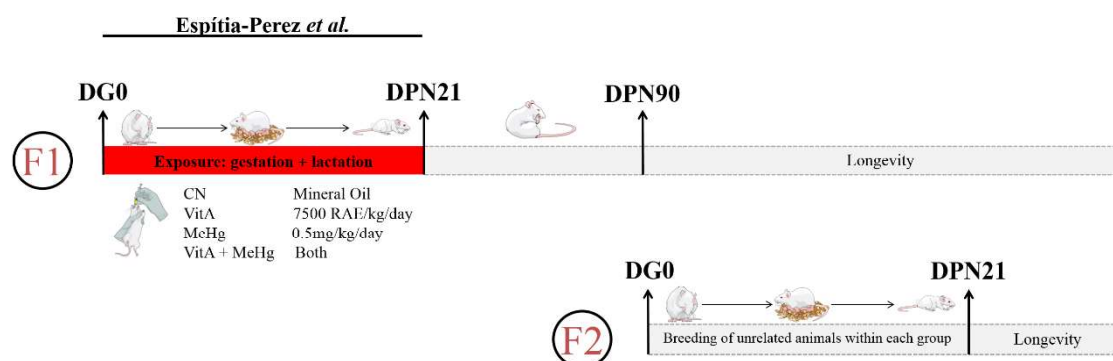
101 of retinyl palmitate (VitA) was prepared at 45000 RAE/ml of mineral oil. Usage solution  
102 for VitA exposure was diluted to 7500 RAE/ml. Females from the MeHg group received  
103 a dose of 0.5 mg of methylmercury (II) chloride per kilogram of body weight each day.  
104 The VitA group received 7500 RAE per kilogram of body weight each day. The co-  
105 exposure group (MeHg+VitA) received the same doses of the previous exposures. All  
106 control group received the vehicle of the MeHg and VitA solutions, mineral oil. None of  
107 the gavages passed the diary volume of 0.5ml. These doses are considered to represent  
108 concentrations to which humans are diary exposed, majorly via fish consumption for  
109 MeHg (Gandhi et al., 2013; Marrugo-Negrete et al., 2013; Obiorah et al., 2015). The VitA  
110 concentration is very far from teratogenic doses, *i.e.* 48900 RAE/kg/day (Ritchie et al.,  
111 1998). However, previous work has already described the toxicological effects of  
112 administration of 7500 RAE/kg/day in dams and offspring (Schnorr et al., 2011a).

### 113 **2.3. Experimental procedures**

114 Experimental procedures are explained in Fig. 1. Is important to note that we repeated  
115 a previous model already conducted by Espítia-Perez *et al.*, from our research group  
116 (Espitia-Perez et al., 2018a; Espitia-Perez et al., 2018b). However, in this work, we made  
117 a generational approach and extended the model until F2. To facilitate reader  
118 comprehension, we summarized the analysis conducted in each generation in Table A.1.

119 Nulliparous females 90 days-old (F0) were mated with males (PND90) for breeding.  
120 The ones with traces of sperm in vaginal lavage were considered as pregnant (GD0) and  
121 separated in four groups: (i) Control (CN); (ii) VitA; (iii) MeHg; and (iv) MeHg+VitA,  
122 *i.e.* co-exposure. Exposures were conducted chronically during 42 days, encompassing the  
123 gestation and lactation periods (from GD0 to PND21). Offspring were defined as F1. F1  
124 rats were separated by sex at PND21 and get old until PND90. At PND90, individuals  
125 from the same group, without parenthood, were mated. No exposure was administered

126 anymore. F1 offspring were defined as F2. Some rats from F2 were euthanized on PND21.  
 127 Rats from F1 and F2 were separated for further longevity analysis (*data not shown*).



128

129 **Figure 1. Experimental overview.** Pregnant Wistar rats (F0) were separated into the  
 130 following groups: (i) Control (CN), which received mineral oil, the vehicle of retinyl  
 131 palmitate (VitA) and methylmercury (MeHg); (ii) VitA, a precursor of vitamin A, at 7500  
 132 RAE/kg/day; (iii) MeHg at 0.5mg/kg/day; and (iv) both VitA and MeHg at the same  
 133 previous concentrations. All treatments were administered by gavage during rat's  
 134 gestation and lactation (~42 days). Part of the offspring (F1) was euthanized for further  
 135 analyses and part for posterior breedings. F1 rats from the same group, without  
 136 parenthood, were mated to generate F2. F2 rats were divided for toxicological and for  
 137 longevity analysis, as well as F1 individuals. RAE, retinol activity equivalents.

138

#### 139 2.4. Reproductive parameters

140 To assess the influence of the exposures we conducted some analyses of reproductive  
 141 success. Bodyweight from dams from F0 and F1 were accompanied during gestation and  
 142 lactation (Fig. A.1 and 2, respectively). The number of uterus implantations and delivered  
 143 pups were counted and used for delivery index calculus (Table 1 for F0 and Table 2 for  
 144 F1), defined the ratio of the number of delivered pups by the number of implantations  
 145 multiplied by one hundred. The sex ratio of the offspring was also accompanied (Table 1



146 for F1 and Table 2 for F2). We also accompanied the pup's weight on PNDs 0, 7, 14, and  
147 21 in F2 (Table 2).

## 148 **2.5. Neurobehavioral analyses**

149 MeHg is a causative agent of Minamata disease (Eto, 2000) and affects  
150 neurodevelopment even in environmental exposures/doses in humans (Al-Saleh et al.,  
151 2016; Engstrom et al., 2016) and rats (Black et al., 2011; Reardon et al., 2019). Thus, we  
152 conducted neurobehavioral assessments in F2 as markers of neurodevelopment (sections  
153 2.5.1 and 2.5.2). These analyses were conducted in F1 previously (Espitia-Perez et al.,  
154 2018b).

### 155 **2.5.1. Maternal odor preference**

156 Based on works regarding associative olfactory learning (2585063, 1487749,  
157 15257129), the maternal odor preference test is a standard for olfactory ability and social  
158 behavior (Rainecki et al., 2009). The test followed the protocol of previous works (Rainecki  
159 et al., 2009; Espitia-Perez et al., 2018b).

### 160 **2.5.2. Eye-opening time**

161 Was already demonstrated the central role of eye-opening in synaptic potentiation  
162 and cortex network activity (Hoy and Niell, 2015; Murata and Colonnese, 2018). As  
163 maternal odor preference, eye-opening time was conducted as previously (Schneider and  
164 Przewlocki, 2005). The eye-opening test was made with all pups from PND12 to 16. The  
165 rate given to pups were: 0 – none of the eyes opened; 1 – One eye opened; and 2 – both  
166 eyes opened.

## 167 **2.6. Organs weight**

168 Organs weight assessments are important for toxicological studies and identification  
169 of harmful effects of compounds (Sellers et al., 2007). In this work, we weighted the *heart*

170 and *kidney* from F1 and F2 at PND21. The same person always extracted organs to avoid  
171 differences in procedures.

## 172 **2.7. Micronucleus frequencies and polychromatic/normochromathic cells ratio**

173 The protocol followed OECD guidelines (OECD, 2014) and was conducted as  
174 previously described (Espitia-Perez et al., 2018a; Rosa-Silva et al., 2019). Bone marrow  
175 was extracted from each F2 PND21 pup's femur. Polychromatic and normochromatic  
176 erythrocytes cells (PCE and NCE, respectively) relative numbers were also evaluated.  
177 Micronuclei frequencies were made per 1000 PCE cells from each femur (MNPCE), *i.e.*  
178 totalizing 2000 PCEs. The presence of high MNPCE frequencies is a biomarker of high  
179 chromosome damage (Mondal et al., 2010). PCE and NCE ratios represent the  
180 hematopoiesis maturation, while the first represent nucleated cell and the latter not. The  
181 change in this ratio is a biomarker of bone marrow toxicity (Suzuki et al., 1989).

## 182 **2.8. Histone 3 trimethylation**

### 183 **2.8.1. Acidic histones enrichment protocol**

184 Extraction and enrichment of histones followed previously established protocols  
185 (Shechter et al., 2007; Beldjoud et al., 2016). Briefly, cerebellum slices were manually  
186 macerated in 100ul of hypotonic lysis buffer (250mM sucrose, Tris 50mM, KCl 25mM,  
187 Sodium butyrate 0.9mM pH 7.5; protease and phosphatase inhibitor cocktail immediately  
188 before usage 1:1000 v/v) and incubated in ice for 5 minutes. Homogenate was centrifuged  
189 at 7600g for 1min, the supernatant was discarded. Pellet was resuspended in 100ul of HCl  
190 0.2M and incubated for 1h in ice, with agitations in vortex every 15min. After incubation,  
191 samples were centrifuged at 16000g for 15min. In a new microtube, proteins from the  
192 supernatant were precipitated in TCA 10% (m/v, final concentration). Samples were  
193 agitated in vortex during 5s and incubated in ice for 15min, they were again centrifuged  
194 at 16000g for 15min. Pellet was washed with 100ul of cold acetone and centrifuged at

195 16000g for 5min. This step was repeated twice. The supernatant was discarded and the  
196 pellet was let dry at room temperature. Pellet were resuspended in 50ul Tris 50mM SDS  
197 3% (m/v) pH 7.5.

### 198 **2.8.2. Immunoblotting**

199 Trimethylation pattern of residues K4, 9, 27, 36 and 79 from histone 3 (H3) was  
200 evaluated in the cerebellum of males from F1 and F2. Monoclonal antibodies from Cell  
201 Signaling Technology (9783T) were utilized for immunoblotting, using 1:1000 dilution.

### 202 **2.9. Statistics**

203 Results were represented as mean  $\pm$  SEM, with the exception in boxplots where  
204 ranges were set as a minimum to maximum. Statistics from Table 3 were calculated in R  
205 environment. Remaining analyses were made in GraphPad Prism 8.0.1 (GraphPad  
206 Software Inc., San Diego, CA, USA). When appropriate, two-way ANOVA was  
207 calculated considering exposures as first factor and day of age as the second for eye-  
208 opening test or sex for organs weight, MNPCE frequencies and PCE/NCE ratios. One-  
209 way and two-way ANOVA were followed by Tukey's *post hoc* test. Significant values  
210 were considered when  $p \leq 0.05$ .

## 211 3. Results

### 212 3.1. Reproductive success data

213 Reproductive parameters of F0 dams and their offspring (F1) are discriminated in  
214 Table 1. A previous work of our group which used the same animal protocol presented  
215 similar data (Espitia-Perez et al., 2018a). The exposures were not able to significantly  
216 influence the number of implantations and delivered pups. Moreover, the sex ratio of  
217 offspring was not affected. However, the delivery index of the MeHg group was  
218 significant lower than the control group but this difference was not seen in co-exposure  
219 group. F1 mortality and malformations were not affected by any of the exposures (data  
220 not shown).

221 Table 2 describes the reproductive parameters of F1 dams and their offspring (F2).  
222 Number of implantations, number of delivered pups, delivery index and sex ratio were  
223 not affected by any of the exposures. However, F2 pups from VitA and co-exposure were  
224 significant lighter than the control group at PND14 and 21. Similarly, individuals from  
225 MeHg were also lighter than control at PND21. F2 mortality and malformations were also  
226 not affected by any of the exposures (data not shown).

**Table 1**

Effects of MeHg, VitA and co-exposure in reproductive parameters from F0. (n=9-28,  
per group)

	Treatments			
	Control	MeHg	VitA	MeHg + VitA
<b>N° of implantations</b>	11 ± 0.78	10.75 ± 0.64	9.39 ± 0.98	10.69 ± 0.69
<b>N° of pups delivered (F1)</b>	10 ± 0.72	7.75 ± 0.84	8.85 ± 0.92	9.63 ± 0.65

<b>Delivery index (%)</b>	91.36 ± 2.19	<b>71.23 ± 6.65*</b>	95.40 ± 2.8	90.11 ± 1.82
<b>Sex ratio of pups (F1)</b>	44.15 ± 5.83	45.65 ± 5.00	42 ± 2.56	55.39 ± 5.26

Delivery index (%) = (number of pups delivered/number of implantations) x 100

Sex ratio of pups = number of male pups/total number of pups

Data are represented as mean±SEM. Bold for statistically significant difference

\* p ≤ 0.05 compared to the control group

227

**Table 2**

Effects of MeHg, VitA and co-exposure in reproductive parameters from F1. (n=6-11, per group)

	Treatments			
	Control	MeHg	VitA	MeHg + VitA
<b>N° of implantations</b>	7.56 ± 1.25	9.10 ± 0.89	8.50 ± 1.02	10.18 ± 0.91
<b>N° of pups delivered (F2)</b>	5.57 ± 1.00	7.55 ± 1.16	8 ± 0.98	9.18 ± 0.87
<b>Delivery index (%)</b>	77.00 ± 9.69	77.28 ± 6.65	94.44 ± 2.8	90.28 ± 4.52
<b>Sex ratio of pups (F2)</b>	40.05 ± 9.09	52.89 ± 6.90	40 ± 6.76	45.70 ± 5.17
<b>Pup weight (g)</b>				
PND0	7.42 ± 0.41	7.31 ± 0.14	7.27 ± 0.08	7.48 ± 0.22
PND7	20.53 ± 0.65	18.5 ± 0.62	17.89 ± 0.67	17.84 ± 0.74
PND14	36.01 ± 1.19	31.84 ± 1.68	<b>30.21 ± 1.45*</b>	<b>28.70 ± 1.20**</b>
PND21	57.43 ± 1.81	<b>52.07 ± 2.08*</b>	<b>49.36 ± 2.16***</b>	<b>47.93 ± 2.23****</b>

Data are represented as mean±SEM. Bold for statistically significant difference

\* p ≤ 0.05 compared to the control group

\*\* p ≤ 0.01 compared to the control group

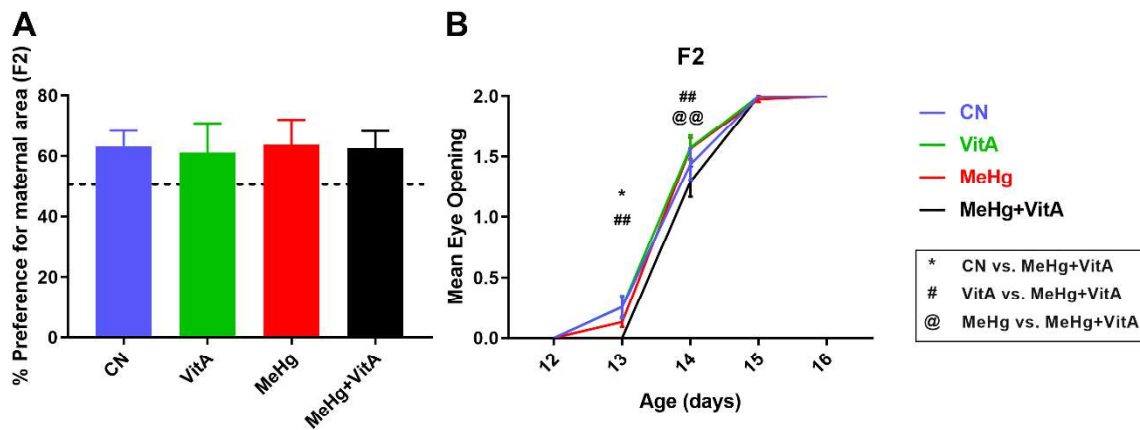
\*\*\* p ≤ 0.001 compared to the control group

\*\*\*\* p ≤ 0.0001 compared to the control group

228

### 3.2. Neurobehavior analyses of F2

229 Direct exposure to MeHg or VitA of dams during gestation and lactation periods  
 230 were not able to affect the preference for maternal bedding in the second generation (Fig.  
 231 2A), in contrast to observed in F1 (Espitia-Perez et al., 2018b). In eye-opening test both  
 232 exposures factor ( $F_{[3, 1135]} = 3.291, p = 0.020$ ) and day of age ( $F_{[4, 1135]} = 1147, p < 0.0001$ )  
 233 were significant. However, the time of eye-opening was delayed by one day in co-  
 234 exposure when compared to control or VitA (Fig. 2B – Day 13). In the PND14, the mean  
 235 eye-opening score of co-exposure was significant lower than the MeHg group. In F1,  
 236 MeHg exposure delayed the time of eye-opening compared to control (Espitia-Perez et  
 237 al., 2018b).



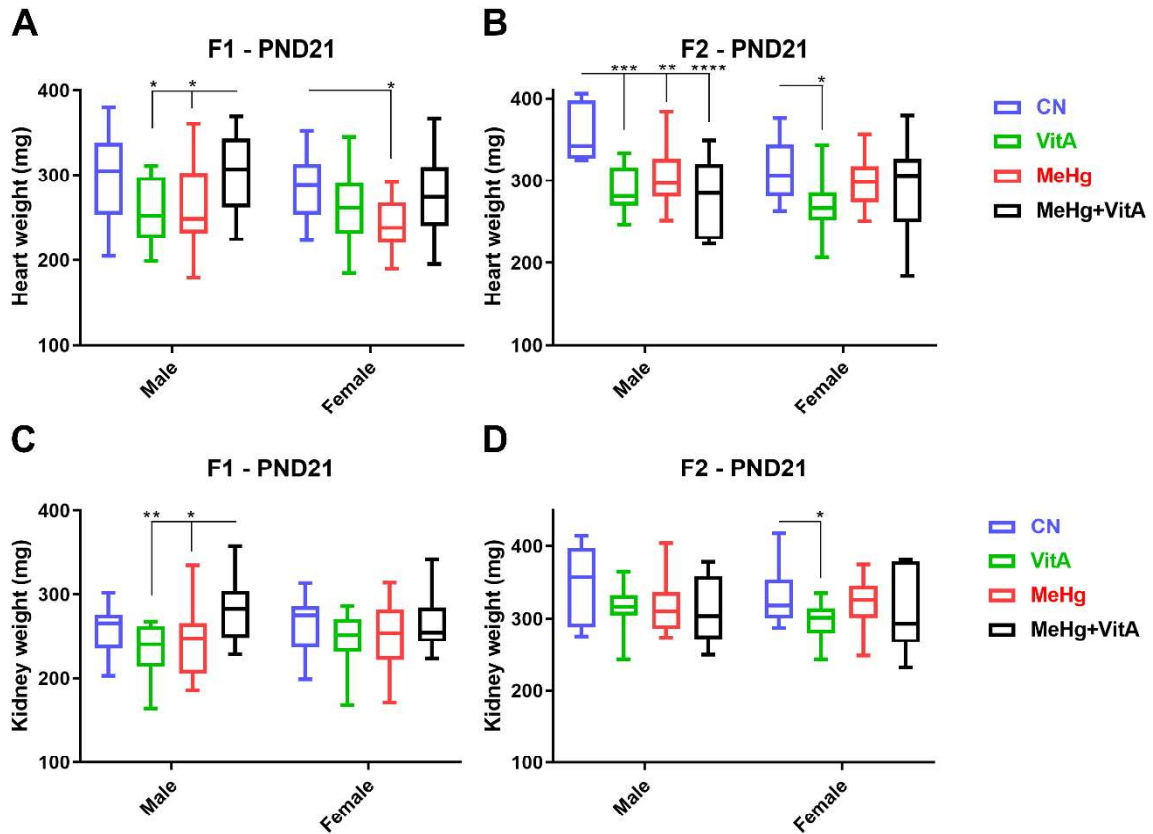
238 **Figure 2. Neurobehavioral analyses of F2.** Maternal odor preference at PND8 (A) and  
 239 time for eye-opening (B). The dotted line indicates the probability of a pup to choose  
 240 bedding randomly. \*  $p \leq 0.05$  when compared to CN; ##  $p \leq 0.01$  when compared to VitA  
 241 exposure; @@  $p \leq 0.01$  when compared to MeHg exposure. CN, control; VitA, retinyl  
 242 palmitate exposure; MeHg, methylmercury exposure; MeHg+VitA, co-exposure of VitA  
 243 and MeHg. (n for the preference of maternal area=6-7, per group; n for eye-opening=39-  
 244 83, per group)

### 246 3.3. Toxicological data

#### 247 3.3.1. Organs weight

248           Once heart and kidney weights are not well modeled by body or brain weights  
249 (Bailey et al., 2004), they were represented as their absolute mass (Fig. 3). MeHg and  
250 VitA exposures affect organs weight differently in males and females, both in F1 and F2.  
251 In F1 males, hearts from MeHg and VitA were lighter than co-exposure ones, which were  
252 not significantly different from control. Furthermore, MeHg exposures were capable of  
253 significantly to reduce the heart weight of females from F1 (Fig. 3A). In F2 males, heart  
254 weight from all exposures was reduced. However, in F2 females this occurred only in  
255 VitA individuals (Fig. 3B).

256           Kidney weight from F1 and F2 were also affected by exposures sex-specifically.  
257 While F1 female's kidneys weight was not altered, VitA and MeHg exposures  
258 significantly reduced it when compared to co-exposure (Fig. 3C). The kidney weight  
259 was not affected by any exposure in F2 males. In contrast, VitA significantly reduced  
260 kidney weight in F2 females when compared to control (Fig. 3D). Is important to note  
261 that every significance encountered in kidney weight was also encountered in hearts. F  
262 statistics were as following: all exposures effects were significative, heart weight F1 ( $F_{[3, 115]} = 7.142, p = 0.0002$ ), heart weight F2 ( $F_{[3, 110]} = 10.13, p < 0.0001$ ), kidney weight F1  
263 ( $F_{[3, 116]} = 5.150, p = 0.0022$ ), kidney weight F2 ( $F_{[3, 94]} = 3.267, p = 0.0247$ ); however sex  
264 effects were not, heart weight F1 ( $F_{[1, 115]} = 2.575, p = 0.1113$ ), heart weight F2 ( $F_{[1, 110]} =$   
265  $3.687, p = 0.0577$ ), kidney weight F1 ( $F_{[1, 116]} = 0.1193, p = 0.7304$ ), kidney weight F2  
266 ( $F_{[1, 94]} = 0.7868, p = 0.3773$ ).



268

269 **Figure 3. Organs weight from F1 and F2 separated by sex at PND21.** Hearts weight  
 270 from F1 (A) and F2 (B). Kidneys weight from F1 (A) and F2 (B). \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ;  
 271 \*\*\*  $p \leq 0.001$ ; and \*\*\*\*  $p \leq 0.0001$ . CN, control; VitA, retinyl palmitate exposure; MeHg,  
 272 methylmercury exposure; MeHg+VitA, co-exposure of VitA and MeHg. (n for F1=8-20,  
 273 per group; n for F2=7-20, per group)



274

Table 3

Effects of MeHg, VitA and co-exposure in MNPCE frequencies and PCE/NCE cells ratios from F2.

N°	Treatments											
	Control			MeHg			VitA			MeHg + VitA		
	Mean ±SEM	Median	25th-75th percentile	Mean ±SEM	Median	25th-75th percentile	Mean ±SEM	Median	25th-75th percentile	Mean ±SEM	Median	25th-75th percentile
MNPCE/2000 cells												
Male	0.35 ± 0.20	0 (0 - 0.25)	0.96 ± 0.39	0.5(0 - 1.14)	0.59 ± 0.43	0 (0 - 0)	1.00 ± 0.36	0.5 (0 - 1.27)				
Female	0.46 ± 0.18	0 (0 - 1.19)	<b>2.04 ± 0.51*</b>	2 (0.25 - 2.69)	0.71 ± 0.25	0 (0 - 1.29)	<b>2.32 ± 0.52**</b>	2.32 (0.34 - 3.58)				
Total	0.41 ± 0.13	0 (0 - 1)	<b>1.50 ± 0.33*</b>	1 (0 - 2.44)	0.65 ± 0.25	0 (0 - 1.04)	<b>1.66 ± 0.33*</b>	1.17 (0 - 3.00)				
PCE/NCE												
Male	2.79 ± 0.28	2.54 (1.94 - 3.52)	<b>4.01 ± 0.25***</b>	4.05 (3.21 - 4.35)	<b>3.93 ± 0.20**</b>	3.59 (3.16 - 4.42)	2.73 ± 0.14	2.83 (2.60 - 2.93)				
Female	2.91 ± 0.19	2.79 (2.40 - 3.36)	3.67 ± 0.21	3.20 (2.74 - 3.82)	<b>3.35 ± 0.20*</b>	3.79 (3.40 - 4.31)	2.99 ± 0.15	3.09 (2.66 - 3.32)				
Total	2.86 ± 0.16	2.79 (2.21 - 3.38)	<b>3.62 ± 0.15**</b>	3.75 (2.92 - 4.18)	<b>3.75 ± 0.16***</b>	3.75 (3.21 - 4.33)	2.87 ± 0.10	2.91 (2.58 - 3.17)				

Data are represented as mean±SEM. Bold for statistically significant difference

\* p ≤ 0.05 compared to the control group

\*\* p ≤ 0.01 compared to the control group

\*\*\* p ≤ 0.001 compared to the control group

275 **3.3.2. MNPCE and PCE/NCE ratios**

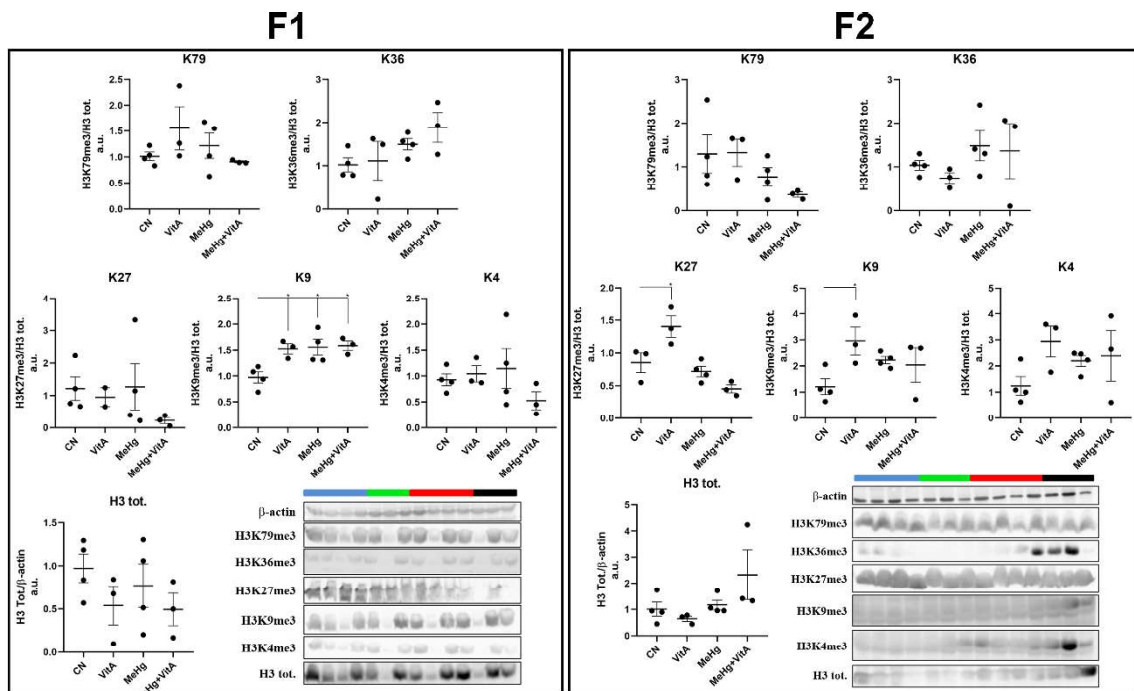
276 Once MNPCE were already demonstrated in F1 (Espitia-Perez et al., 2018a), Table  
 277 3 reveals the influence of MeHg and VitA at chronic damage do DNA in F2. In MNPCE

278 frequencies, both exposure ( $F_{[3, 102]} = 5.220$ ,  $p = 0.0022$ ) and sex ( $F_{[1, 102]} = 5.955$ ,  $p =$   
279  $0.0164$ ) factors were significant. However, sex factor was not significant for  
280 PCE/NCE ratios ( $F_{[1, 102]} = 0.3406$ ,  $p = 0.5608$ ), while exposure factor was ( $F_{[3, 102]} =$   
281  $11.21$ ,  $p < 0.0001$ ). While MNPCE frequencies of VitA were at control levels in F2,  
282 MeHg and co-exposure increased significantly this frequency in females and both sexes  
283 when considered together. The increase of MNPCE in co-exposure is probably due to the  
284 effect of MeHg alone. As a biomarker of bone marrow toxicity, the PCE/NCE ratio was  
285 increased in males and both sexes in the MeHg group. Moreover, males and females from  
286 the VitA group have an increased ratio too when compared to control. Interestingly, no  
287 effect of PCE/NCE ratio was observed in co-exposure.

### 288 **3.4. H3 trimethylations**

289 Despite the signal fluctuation, the immunocontent of H3 was not significantly  
290 affected by exposures neither in F1 nor F2, when normalized by  $\beta$ -actin (Fig. 4). Previous  
291 work from our laboratory demonstrated that MeHg exposure during neurodevelopment  
292 leads to redox imbalance and ERK1/2 and JNK pathways disruption in the cerebellum  
293 (Heimfarth et al., 2018b). Considering these results, we assessed the H3 trimethylation  
294 pattern in the cerebellum to elucidate if there is a putative epigenetic modulation in this  
295 tissue.

296 In F1, only the trimethylation of H3K9 was significantly increased as a result of  
297 exposures. Moreover, in F2 both H3K9 and H3K27 trimethylation were increased in the  
298 VitA group. Regarding the present data, MeHg alone seems not to affect the  
299 trimethylation pattern of H3 in F2.



300

301 **Figure 4. Histone 3 trimethylation pattern in F1 and F2 from the male cerebellum.**

302 \*  $p \leq 0.05$ . Blue for CN, control; Green for VitA, retinyl palmitate exposure; Red for

303 MeHg, methylmercury exposure; Black for MeHg+VitA, co-exposure of VitA and

304 MeHg. (n=3-4, per group)

305

#### 306 4. Discussion

307 Is already well demonstrated in the literature that contact to MeHg during gestation  
308 leads to neurodevelopment and cognitive deficits in children (Grandjean et al., 1997;  
309 Boucher et al., 2012; Julvez et al., 2013). High doses of vitamin A also are prejudicial to  
310 health and can lead to teratogenesis and congenital defect in fetuses (Azais-Braesco and  
311 Pascal, 2000). With the usage of supplementation pills and disposal of enriched foods in  
312 diet, the diary recommended concentrations of vitamin A can be exceeded, including  
313 pregnant women. Moreover, fish consumption, an important source of both MeHg and  
314 VitA, is increasing in the last years by pregnant (Razzaghi and Tinker, 2014; Cusack et  
315 al., 2017).

316 Were shown by our group that offspring exposed to MeHg and/or VitA *in utero*  
317 have toxicological effects (Schnorr et al., 2011b; Schnorr et al., 2015; Espitia-Perez et al.,  
318 2018a; Espitia-Perez et al., 2018b; Heimfarth et al., 2018a; Heimfarth et al., 2018b).  
319 Moreover, adulthood re-exposure to MeHg also showed to worsen toxicological  
320 parameters (Rosa-Silva et al., 2019), representing the environmental exposure.  
321 Altogether, there is an increasing demand for a determination of whether these  
322 compounds are modulating mechanisms that perpetuate the injuries. In this context, the  
323 present study showed that exposures to MeHg and VitA can delay eye-opening time (Fig.  
324 2B), affect organs weight (Fig. 3) and enhance chromosome damage (Table 3) in F2.

325 Retinoic acid, the major metabolite of vitamin A, is known to be an important  
326 modulator of cell differentiation and proliferation (Duester, 2008; Connolly et al., 2013;  
327 Draut et al., 2019). Retinol, another vitamin A form, show to play a central role in  
328 epigenetic erasure in pluripotent cells (Hore et al., 2016). Despite that literature have  
329 described the very promising role of vitamin A derivatives in epigenetics, the studies are  
330 focused on cell lines and do not discuss the influence of environmental exposures. As an

331 example, Lee et al. (2007) (Lee et al., 2007) described a decrease of H3K27 trimethylation  
332 in mouse embryonic stem cells. However, the methylation pattern of this residue was not  
333 affected in F1 and was enhanced in F2 (Fig. 4). Moreover, when an acute dose of 90mg/kg  
334 of pregnant rat body weight at GD10 of all-trans retinoic acid was given to induce neural  
335 tube defects, the trimethylation of H3 lysine 27 was also increased in primary neural stem  
336 cells (Zhai et al., 2016).

337         Hou *et al.* (2015) demonstrated that vitamin A deficiency led to learning and  
338 memory impairments in Wistar rats. Moreover, the article also found altered acetylation  
339 pattern of H3 and concluded suggesting the importance to ensure sufficient nutritional  
340 vitamin A during pregnancy (Hou et al., 2015). This together with the present work may  
341 seem conflicting. However, there is a dosage paradigm of vitamin A consumption  
342 (Duerbeck and Dowling, 2012). The lack of vitamin A in diet, majorly during  
343 neurodevelopment, is prejudicial to health and can lead to traits such as blindness (Aghaji  
344 et al., 2019). In the same way, the excess of vitamin A in the diet also affect negatively  
345 health. We showed that high non-teratogenic doses, such as 7500 RAE/kg/day, can also  
346 lead to toxicological effects, including transgenerational inheritance of them. These doses  
347 are capable of change cardiac and kidney weight (Fig. 3) in both generations and enhance  
348 bone marrow toxicity (Table 3) in F2. Our results are in accordance with previous works  
349 which describe deleterious outcomes of high doses of vitamin A (Ritchie et al., 1998;  
350 Schnorr et al., 2011b).

351         The epigenetic influence of methylmercury is yet suggestional but promising. *In*  
352 *vivo*, MeHg showed to alter miRNAs levels (Rudgalvyte et al., 2013; Hu et al., 2017);  
353 change histone modifications (Onishchenko et al., 2008; Guida et al., 2016; Rudgalvyte  
354 et al., 2017); and influence DNA methylation (Desaulniers et al., 2009; Basu et al., 2013;  
355 Olsvik et al., 2014; Khan et al., 2017). Nevertheless, these studies considered only direct

356 exposures to MeHg. Therefore, it could not be considered as epigenetic modulation of  
357 MeHg once epigenetic needs to install transgenerational phenotypes (Wu and Morris,  
358 2001).

359 As described by (Culbreth and Aschner, 2019), few studies evaluated the  
360 transgenerational inheritance effects of methylmercury. In zebrafish, different exposures  
361 times and doses were capable to induce visual defects, neurobehavioral alterations,  
362 hypermethylation of genes in F2 (Olsvik et al., 2014; Xu et al., 2016; Carvan et al., 2017)  
363 and learning impairment in F3 (Xu et al., 2016). Furthermore, periconception exposure  
364 to MeHg+Cadmium in mice altered blood glucose tolerance in F2 and F4 and elevated  
365 abdominal adipose tissue weight in F2, F3 and F4 in males. Bodyweight was also altered  
366 in F2 and F4 (Camsari et al., 2019). This work also determined the pattern of  
367 transgenerational inheritance of toxicological effects. The males from the matrilineal line  
368 were the most affected (Camsari et al., 2019). Despite this is the first and only one study  
369 which describes the transgenerational inheritance effect of MeHg in rodents to date (by  
370 our knowledge), it conducted only the co-exposure with Cadmium. Thus, delineating the  
371 MeHg-specific effects are not possible.

372 Skinner, M.K. (2008) (Skinner, 2008) discusses that the first generation not  
373 directly exposed to environmental transgenerational studies is F3. Considering that the  
374 F2 germline is in development during F1 embryo formation, it may be directly affected  
375 when F0 is exposed to pollutants during pregnancy. However, the concentration of total  
376 mercury in the liver from F1 rats is near to the lower limit of detection (Espitia-Perez et  
377 al., 2018a). Further studies are needed to determine if there is MeHg present in gonadal  
378 from F1 and in tissues from F2. Regardless of this debate, the F1 generation is invariably  
379 considered to be directly exposed (Camsari et al., 2019).

380 Aligned with literature, the present work demonstrates the transgenerational  
381 inheritance effects from the exposure to doses considered safe of MeHg and/or VitA in  
382 rats. These compounds showed to lead to neurobehavioral alteration (Fig. 2B), altered  
383 organs weight both in males and females (Fig. 3), increase chronic DNA damage and  
384 bone marrow toxicity (Table 3) and, finally, change histones trimethylation landscapes  
385 (Fig. 4). As an environmental model, is not possible to determine if the toxicological  
386 effects were inherited matrilineally or patrilineally. Nonetheless, it represents real-life  
387 exposure once is more probable for people to relate with others with the same  
388 environmental background. Furthermore, some effects also showed to have sex-specific  
389 patterns (Fig.3, Table 3), reproducing previous works (Espitia-Perez et al., 2018b; Rosa-  
390 Silva et al., 2019). Is difficult to evaluate if co-exposure of MeHg and VitA has antagonist  
391 or synergist effects. Only co-exposure was capable of induce a delay in the time of eye-  
392 opening in F2 when compared to control (Fig. 2), demonstrating a putative synergic  
393 effect. In contrast, co-exposure elevated hearts and kidneys weight to control levels in  
394 males from F1 (Fig. 3 A and C) or maintained in the same levels of MeHg or VitA in F2  
395 (Fig. 3 B and D), suggesting antagonistic or neutral influence, respectively. In MNPCE  
396 frequencies, co-exposure followed the profile of only MeHg, indicating that just MeHg  
397 triggered severe DNA damage in this model.

398 Environmental poisoning by MeHg is gaining public attention. In the last years  
399 not just report cases from acute exposures like Minamata (Harada, 1995; Yorifuji et al.,  
400 2011) are growing in literature. Studies with humans describes also neurodevelopment  
401 impairments and cognitive deficits in a child exposed environmentally during gestation  
402 and lactation in the Republic of Seychelles (Llop et al., 2017b; Irwin et al., 2019), Saudi  
403 Arabia (Al-Saleh et al., 2016) and United Kingdom (Julvez et al., 2019), three continent-  
404 distinct countries. Moreover, autochthonous communities are also affected, such as

405 Yanomami people from Amazon, Brazil, which are consuming contaminated food by  
406 inappropriate discard of mercury from irregular gold mining companies (Vega et al.,  
407 2018).

408 Generational models are well fit to determine putative epigenetic modulation of a  
409 given compound. Our data reinforce the hypothesis that MeHg and VitA may be agents  
410 of transgenerational inheritance of toxicological parameters. With the increasing  
411 knowledge, the transgenerational inheritance of MeHg-induced epigenetic modification  
412 is less and less conceivable, as typified in (Culbreth and Aschner, 2019), and more and  
413 more an environmental reality.

414 The main limitations of this work were (i) not conduct the experiments to further  
415 generations and (ii) not contribute to the discussion if the inheritance is majorly  
416 matrilineal or patrilineal, once individuals from F1 had the same exposure background.  
417 In another way, the protocol followed represents more faithfully real-life exposures, *i. e.*  
418 people tend to relate with other people with the same environmental background.  
419 Moreover, people in high exposed areas of MeHg and/or VitA probably have contact with  
420 these compounds not just during gestation but also during adulthood. Another work from  
421 our laboratory already determined that rats double exposed in neurodevelopment and  
422 adulthood are more fragile (Rosa-Silva et al., 2019).



423 **5. Conclusion**

424 To our knowledge, this is the first study that describes the transgenerational  
425 inheritance effects of the association of MeHg and VitA in rats in an environmental-based  
426 model. The toxicological effects were not extinguished until F2, suggesting that more  
427 generations may be affected. MeHg and coexposure groups were capable affected H3K9  
428 trimethylation pattern in F1 and VitA group affected both in F1 and F2. Moreover, VitA  
429 group also showed an hypermethylation of H3K27 in F2 when compared do control.  
430 Altogether, these data reinforce the hypothesis that MeHg and VitA are modulating  
431 epigenetics. Our work signalizes the urge for competent agents to take remedial policies  
432 to avoid future next generations' health problems.

433

434

435 *Ethical standards and approval*

436 This work has not any clinical data with humans performed by any of the authors.  
437 All experiments conducted are under with national and international practices. The Usage  
438 of Animals Ethics Commission (CEUA) of the Universidade Federal do Rio Grande do  
439 Sul has approved this study – project number 34886.

440 *Conflict of interests*

441 All authors declare to have no conflict of interest.

442 **Highlights**

- 443 • Methylmercury and vitamin A perpetuate injuries by transgenerational inheritance
- 444 • Vitamin A precursor showed to affect histone methylation of F1 and F2
- 445 • Methylmercury affected histone methylation in F1
- 446 • Methylmercury increase DNA chronic damage of F2
- 447 • Environmental exposure to methylmercury and vitamin A lead to health problem

448 **Author Contributions**

449 **FGCK:** Conceptualization, Data curation, Formal analysis, Investigation,  
450 Methodology, Project administration, Validation, Writing - original draft, Visualization;  
451 **MSC:** Data curation, Formal analysis, Investigation, Validation, Writing - original draft;  
452 **HTRS:** Formal analysis, Investigation, Methodology; **PB:** Formal analysis, Investigation,  
453 Writing - review & editing; **AKS:** Formal analysis, Investigation, Writing - review &  
454 editing; **AAT:** Formal analysis, Writing - review & editing; **CTR:** Formal analysis; **DOP:**  
455 Formal analysis; **LSS:** Formal analysis, Investigation; **GA:** Formal analysis; **DPG:**  
456 Funding acquisition, work hypothesis, Resources, Writing - review & editing; **JCFM:**  
457 Conceptualization, Funding acquisition, Methodology, Project administration,  
458 Resources, Supervision, Writing - original draft, Writing - review & editing.

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464 authors declare to approve the present work.

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## DISCUSSÃO

Até nosso conhecimento, esse trabalho é o pioneiro em demonstrar os efeitos toxicológicos herdáveis, isolados ou em coexposição, causados por MeHg e palmitato de retinol (VitA) em ratos. Considerando os resultados apresentados, o MeHg foi capaz de afetar o padrão de metilação de histonas em animais machos da F1. Enquanto isso a VitA foi capaz de afetar tanto a F1 quanto a F2. Assim, a hipótese de que tais compostos estão modulando o perfil toxicológico de maneira epigenética foi confirmada. Isso se dá não só no perfil de modificação de histonas, mas também, e principalmente, pelo padrão de herança das modificações.

Diversos estudos com coortes humanas já avaliaram o impacto do MeHg no neurodesenvolvimento de crianças após serem expostas durante a gestação<sup>25; 27; 47; 48</sup>. Além disso, a exposição ao poluente já está afetando inclusive comunidades tradicionais<sup>49</sup>. Assim, o nosso estudo sugere que possivelmente as próximas gerações também possam ter a sua saúde afetada. Contudo, isso se considera para um caso em que a exposição não é continuada, ou seja, que a criança exposta *in utero* não tenha mais contato com MeHg durante a vida. Sabemos que essa re-exposição não atenua os danos; então, os efeitos toxicológicos podem ser perpetrados de uma maneira ainda mais contundente. A re-exposição ao MeHg durante a fase adulta mostrou intensificar o dano à saúde em ratos, segundo um artigo recentemente publicado por nosso grupo de pesquisa<sup>50</sup>.

Como perspectivas, pretendemos ampliar a análise de marcadores epigenéticos. Estender o perfil de metilação de histonas para fêmeas e avaliar o padrão de metilação de DNA. Além disso, pretendemos avaliar marcadores séricos de danos hepáticos e renais, como as atividades de AST e ALT, concentração de ureia e creatinina. Uma vez que os efeitos toxicológicos não foram extinguidos até a F2, em um futuro projeto se pretende ampliar o número de gerações avaliadas. Atualmente estamos conduzindo uma análise de longevidade com animais da F1 e F2 e pretendemos incluir tal resultado no artigo em preparação assim que o estudo seja finalizado.

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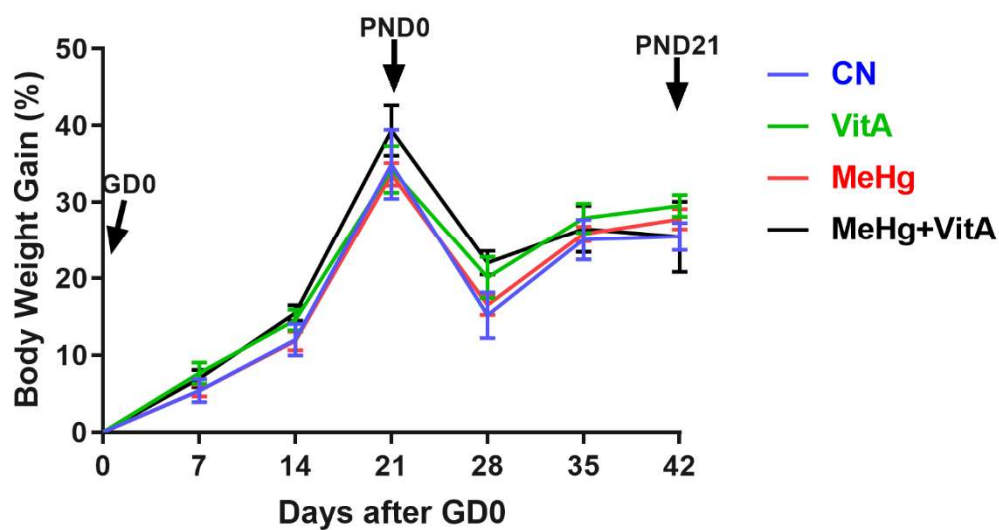
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## ANEXO 1 – APPENDIX A

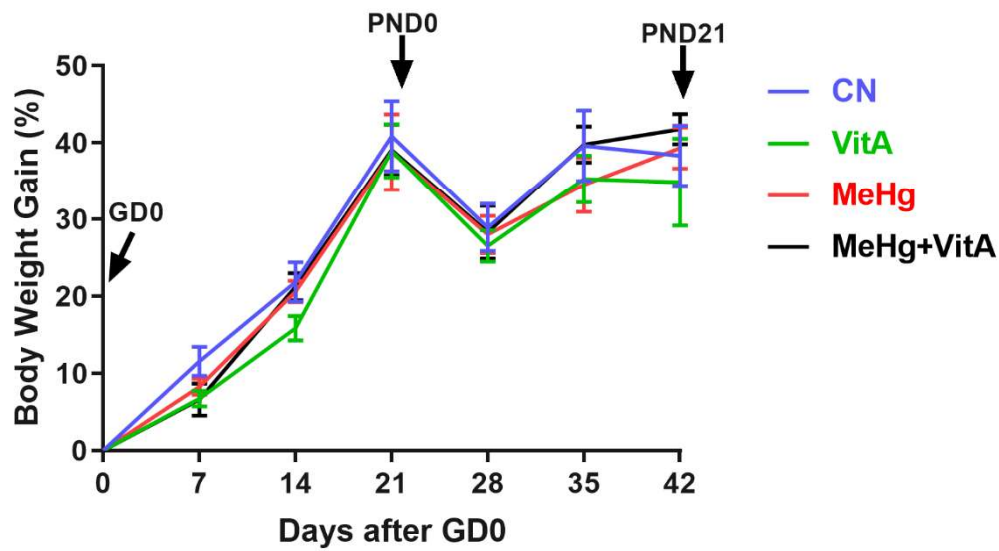
**Table A.1**

Summarization of analysis conducted in each generation

	F1	F2
Reproductive parameters	Table 1	Table 2
Maternal odor preference	Espítia-Perez (2018b)	Fig. 2A
Eye-opening time	Espítia-Perez (2018b)	Fig. 2B
Heart weight	Fig. 3A	Fig. 3B
Kidney weight	Fig. 3C	Fig. 3C
MNPCE frequencies	Espítia-Perez (2018a)	Table 3
PCE/NCE ratios	-	Table 3
Histone 3 trimethylation pattern (cerebellum tissue, male rats)	Fig. 4	Fig. 4

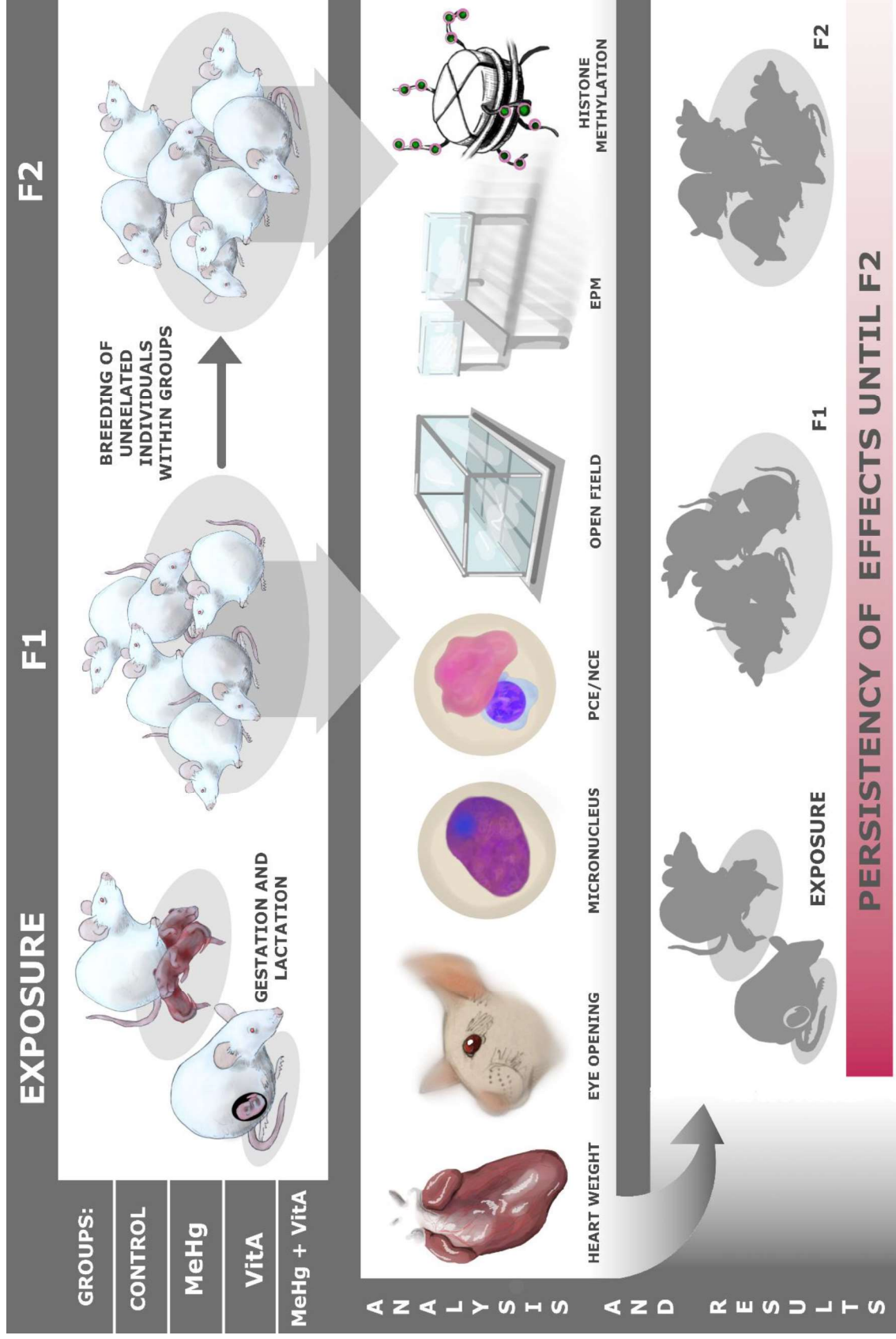


**Fig. A.1. Body weight gain of F0 pregnant rats during gestation and lactation.** The first weight (GD0) was used as reference for body weight gain calculus. Example of body weight gain calculus on GD7: (body weight on GD7/body weight on GD0) \* 100.



**Fig. A2. Body weight gain of F1 pregnant rats during gestation and lactation.** The first weight (GD0) was used as reference for body weight gain calculus. Example of body weight gain calculus on GD7: (body weight on GD7/body weight on GD0) \* 100.

## **ANEXO 2 – FIGURAS E TABELAS**



**Graphical Abstract.** After exposure during gestation and lactation to MeHg and/or VitA, toxicological and epigenetic analysis were conducted with F1 and F2. Altered parameters were found in rats from each group in both generations.



**Table 1**

Effects of MeHg, VitA and co-exposure in reproductive parameters F0. (n = 9-28, per group)

	Treatments			
	Control	MeHg	VitA	MeHg + VitA
<b>N° of implantations</b>	11 ± 0.78	10.75 ± 0.64	9.39 ± 0.98	10.69 ± 0.69
<b>N° of pups delivered (F1)</b>	10 ± 0.72	7.75 ± 0.84	8.85 ± 0.92	9.63 ± 0.65
<b>Delivery index (%)</b>	91.36 ± 2.19	<b>71.23 ± 6.65*</b>	95.40 ± 2.8	90.11 ± 1.82
<b>Sex ratio of pups (F1)</b>	44.15 ± 5.83	45.65 ± 5.00	42 ± 2.56	55.39 ± 5.26

Delivery index (%) = (number of pups delivered/number of implantations) x 100

Sex ratio of pups = number of male pups/total number of pups

Data are represented as mean±SEM. Bold for statistically significant difference

\* p ≤ 0.05 compared to the control group

**Table 2**

Effects of MeHg, VitA and co-exposure in reproductive parameters of F1. (n = 6-11, per group)

	Treatments			
	Control	MeHg	VitA	MeHg + VitA
<b>N° of implantations</b>	7.56 ± 1.25	9.10 ± 0.89	8.50 ± 1.02	10.18 ± 0.91
<b>N° of pups delivered (F2)</b>	5.57 ± 1.00	7.55 ± 1.16	8 ± 0.98	9.18 ± 0.87
<b>Delivery index (%)</b>	77.00 ± 9.69	77.28 ± 6.65	94.44 ± 2.8	90.28 ± 4.52
<b>Sex ratio of pups (F2)</b>	40.05 ± 9.09	52.89 ± 6.90	40 ± 6.76	45.70 ± 5.17
<b>Pup weight (g)</b>				
PND0	7.42 ± 0.41	7.31 ± 0.14	7.27 ± 0.08	7.48 ± 0.22
PND7	20.53 ± 0.65	18.5 ± 0.62	17.89 ± 0.67	17.84 ± 0.74
PND14	36.01 ± 1.19	31.84 ± 1.68	<b>30.21 ± 1.45*</b>	<b>28.70 ± 1.20**</b>
PND21	57.43 ± 1.81	<b>52.07 ± 2.08*</b>	<b>49.36 ± 2.16***</b>	<b>47.93 ± 2.23****</b>

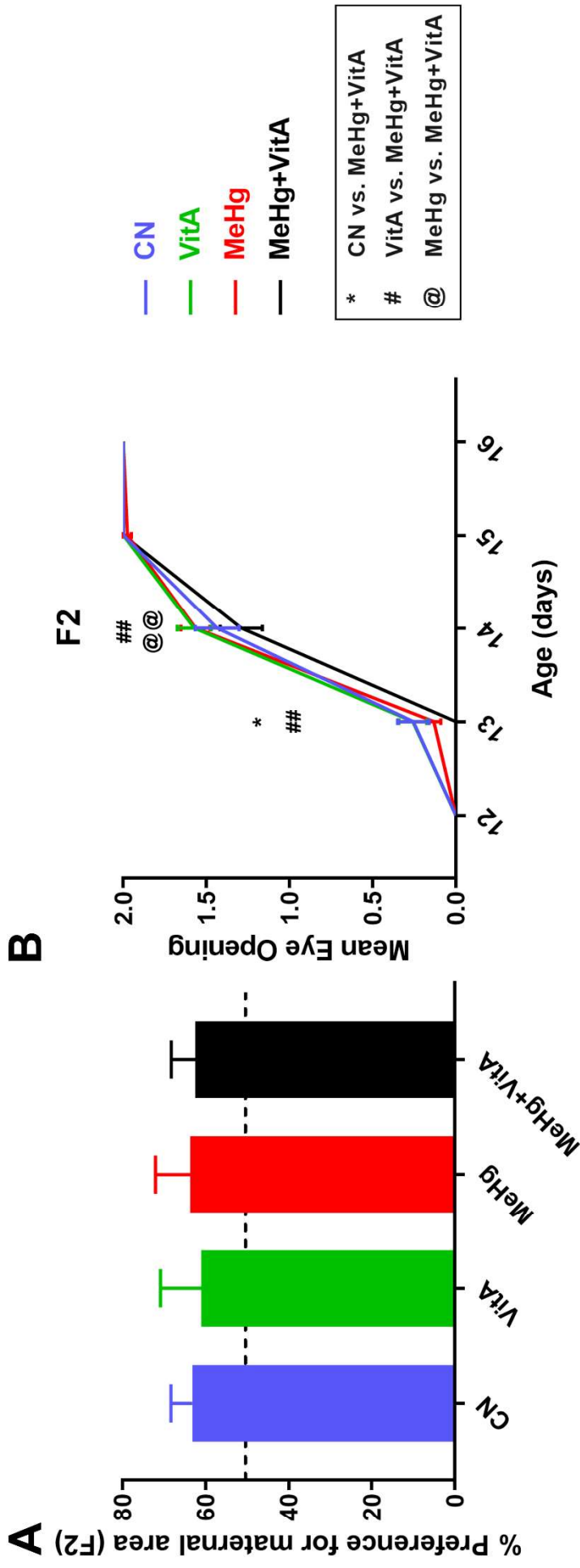
Data are represented as mean±SEM. Bold for statistically significant difference

\* p ≤ 0.05 compared to the control group

\*\* p ≤ 0.01 compared to the control group

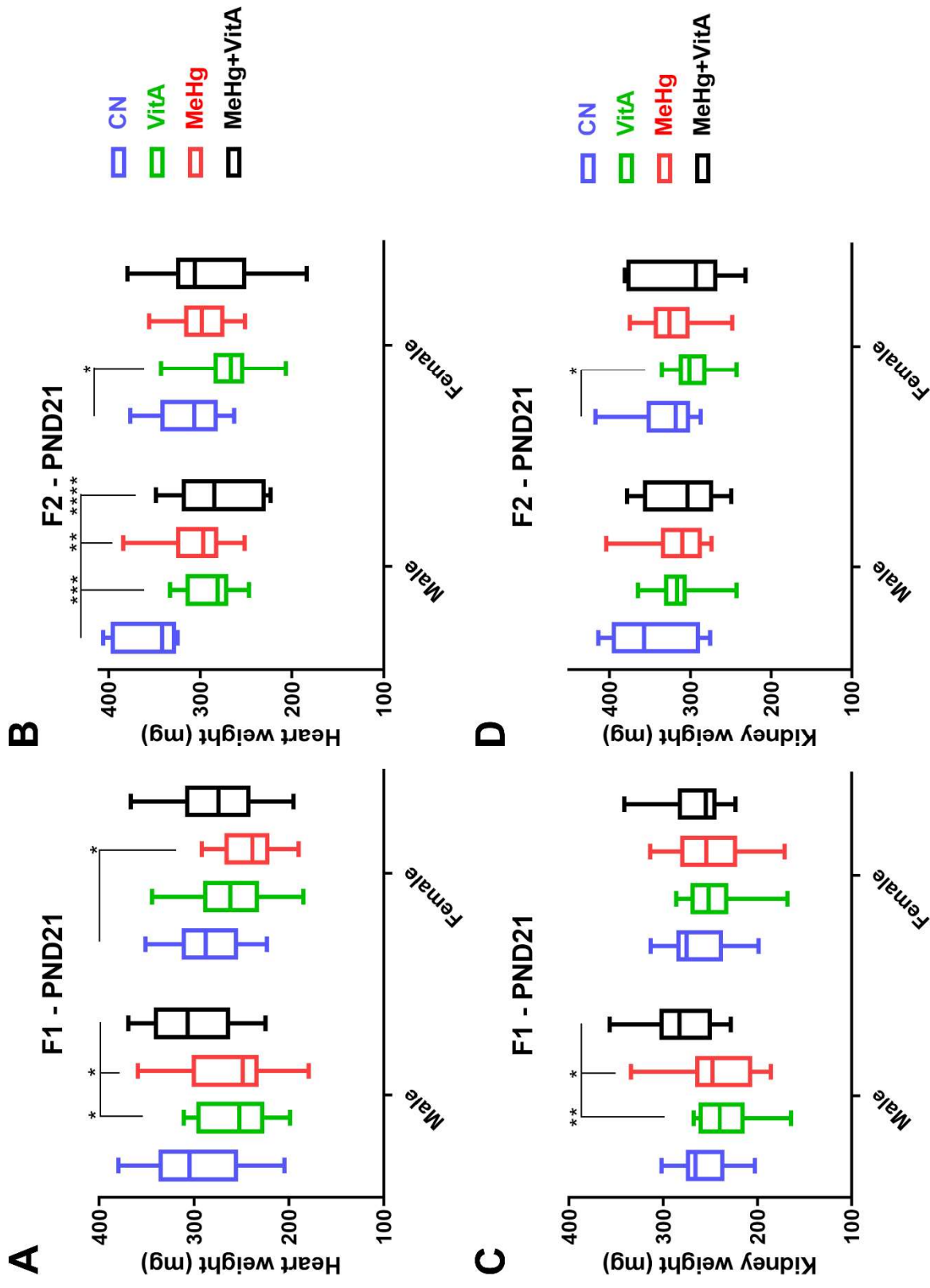
\*\*\* p ≤ 0.001 compared to the control group

\*\*\*\* p ≤ 0.0001 compared to the control group



**Figure 2. Neurobehavioral analyses of F2.** Maternal odor preference at PND8 (A) and time for eye-opening (B). The dotted line indicates the probability of a pup to choose bedding randomly. \*  $p \leq 0.05$  when compared to CN; #  $p \leq 0.01$  when compared to Vita exposure; @  $p \leq 0.01$  when compared to MeHg exposure. CN, control; Vita, retinyl palmitate exposure; MeHg, methylmercury exposure; MeHg+Vita, co-exposure of Vita and MeHg. (n for the preference of maternal area=6-7, per group; n for eye-opening=39-83, per group)





**Figure 3. Organs weight from F1 and F2 separated by sex at PND21.** Hearts weight from F1 (A) and F2 (B). Kidneys weight from F1 (C) and F2 (D). \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; and \*\*\*\*  $p \leq 0.0001$ . CN, control; VitA, retinyl palmitate exposure; MeHg, methylmercury exposure; MeHg+VitA, co-exposure of VitA and MeHg. (n for F1=8-20, per group; n for F2=7-20, per group)

**Table 3**

Effects of MeHg, VitA and co-exposure in MNPCE frequencies and PCE/NCE cells ratios from F2.

N°	Treatments											
	Control			MeHg			VitA			MeHg + VitA		
	7	7	7	7	7	7	7	7	7	7	7	7
MNPCE/2000 cells	Mean ±SEM	Median 25th-75th percentile	Mean ±SEM	Median 25th-75th percentile	Mean ±SEM	Median 25th-75th percentile	Mean ±SEM	Median 25th-75th percentile	Mean ±SEM	Median 25th-75th percentile	Mean ±SEM	Median 25th-75th percentile
Male	0,35 ± 0,20	0 (0 - 0,25)	0,96 ± 0,39	0,5(0 - 1,14)	0,59 ± 0,43	0 (0 - 0)	1,00 ± 0,36	0 (0 - 0)	0,5 (0 - 1,27)			
Female	0,46 ± 0,18	0 (0 - 1,19)	<b>2,04 ± 0,51*</b>	2 (0,25 - 2,69)	0,71 ± 0,25	0 (0 - 1,29)	<b>2,32 ± 0,52**</b>	0 (0 - 1,29)	2,32 (0,34 - 3,58)			
Total	0,41 ± 0,13	0 (0 - 1)	<b>1,50 ± 0,33*</b>	1 (0 - 2,44)	0,65 ± 0,25	0 (0 - 1,04)	<b>1,66 ± 0,33*</b>	0 (0 - 1,04)	1,17 (0 - 3,00)			
<b>PCE/NCE</b>												
Male	2,79 ± 0,28	2,54 (1,94 - 3,52)	<b>4,01 ± 0,25***</b>	4,05 (3,21 - 4,35)	<b>3,93 ± 0,20**</b>	3,59 (3,16 - 4,42)	2,73 ± 0,14	3,59 (3,16 - 4,42)	2,83 (2,60 - 2,93)			
Female	2,91 ± 0,19	2,79 (2,40 - 3,36)	3,67 ± 0,21	3,20 (2,74 - 3,82)	<b>3,35 ± 0,20*</b>	3,79 (3,40 - 4,31)	2,99 ± 0,15	3,79 (3,40 - 4,31)	3,09 (2,66 - 3,32)			
Total	2,86 ± 0,16	2,79 (2,21 - 3,38)	<b>3,62 ± 0,15**</b>	3,75 (2,92 - 4,18)	<b>3,75 ± 0,16***</b>	3,75 (3,21 - 4,33)	2,87 ± 0,10	3,75 (3,21 - 4,33)	2,91 (2,58 - 3,17)			

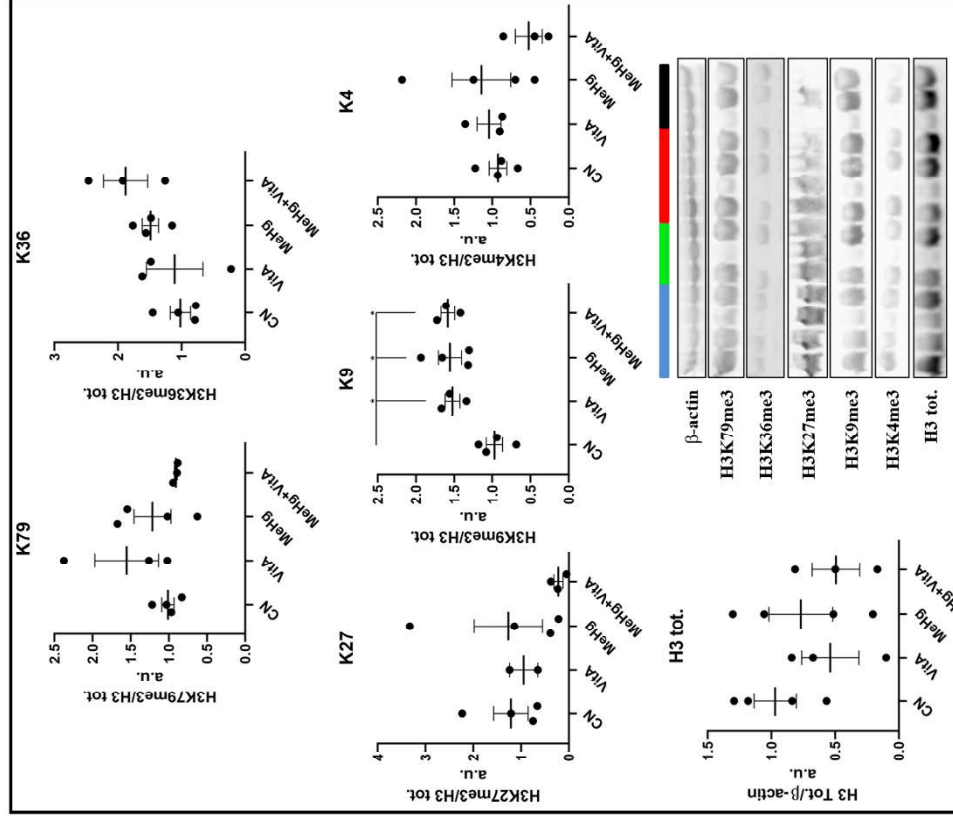
Data are represented as mean±SEM. Bold for statistically significant difference

\* p ≤ 0.05 compared to the control group

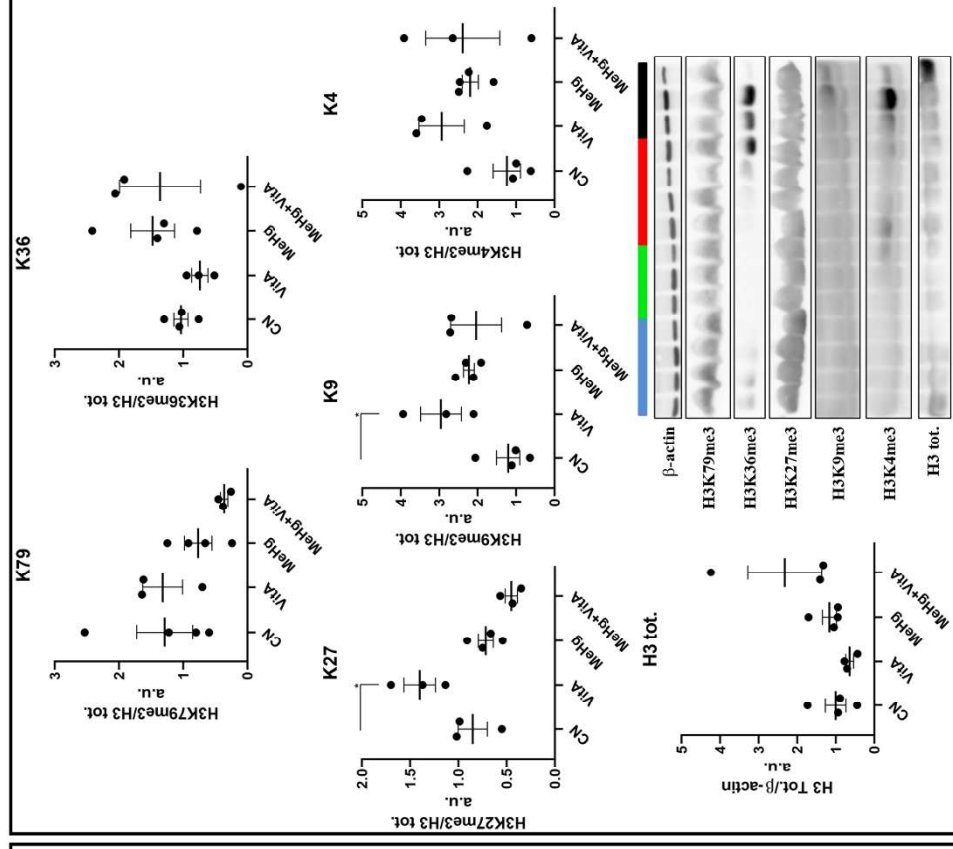
\*\* p ≤ 0.01 compared to the control group

\*\*\* p ≤ 0.001 compared to the control group

F1



F2



**Figure 4. Histone 3 trimethylation pattern in F1 and F2 from the male cerebellum.** \*  $p \leq 0.05$ . Blue for CN, control; Green for VitA, retinyl palmitate exposure; Red for MeHg, methylmercury exposure; Black for MeHg+VitA, co-exposure of VitA and MeHg. (n=3-4, per group)